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Anti-biofilm activity of a polysaccharide from marine sponge associated *Bacillus licheniformis*

By

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Dedication

I would like to dedicate this work to my beloved father, Md. Sekander Ali Miah, to whom I am always indebted.

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Abstract

Secondary metabolites ranging from furanone to exo-polysaccharides have been suggested to have anti-biofilm activity in various recent studies. Among these, *Escherichia coli* group II capsular polysaccharides were shown to inhibit biofilm formation in a wide range of organisms and more recently marine *Vibrio* sp. and *Kingella kingae* were found to secrete complex exopolysaccharides having the potential for broad-spectrum biofilm inhibition and disruption.

In this study, a ca. 1800 kDA polysaccharide having simple monomeric units of α-D-galactopyranosyl-(1→2)-glycerol-phosphate was found to exert an anti-biofilm activity against both Gram-positive and Gram-negative bacteria without any bactericidal effect. This polysaccharide was extracted from a *Bacillus licheniformis* strain associated with the Mediterranean marine organism *Spongia officinalis*. Unlike most of the anti-biofilm compounds, the mechanism of action of the compound was most likely independent from quorum sensing, as its structure is unrelated to any of the so far known quorum sensing molecules. This was unexpected, since previous studies had shown that the combined action of α-D-galactopyranosyl-glycerol (floridoside) and isethionic acid (floridoside-isethionic acid complex) from red algae had anti-biofilm effect through quorum sensing inhibition.

Other experiments revealed that treatment of abiotic surfaces with the present polysaccharide blocked and/or reduced the initial adhesion and biofilm development of strains such as *Escherichia coli* PHL628 and *Pseudomonas fluorescens*. In addition the polysaccharide reduced the cell surface hydrophobicity of the tested strains which appeared to be a cause of reduction of cell-cell or cell-surface interaction during the initial attachment stage of biofilm development.

Further research on such surface-active compounds might help in developing novel and more potential anti-biofilm molecules.

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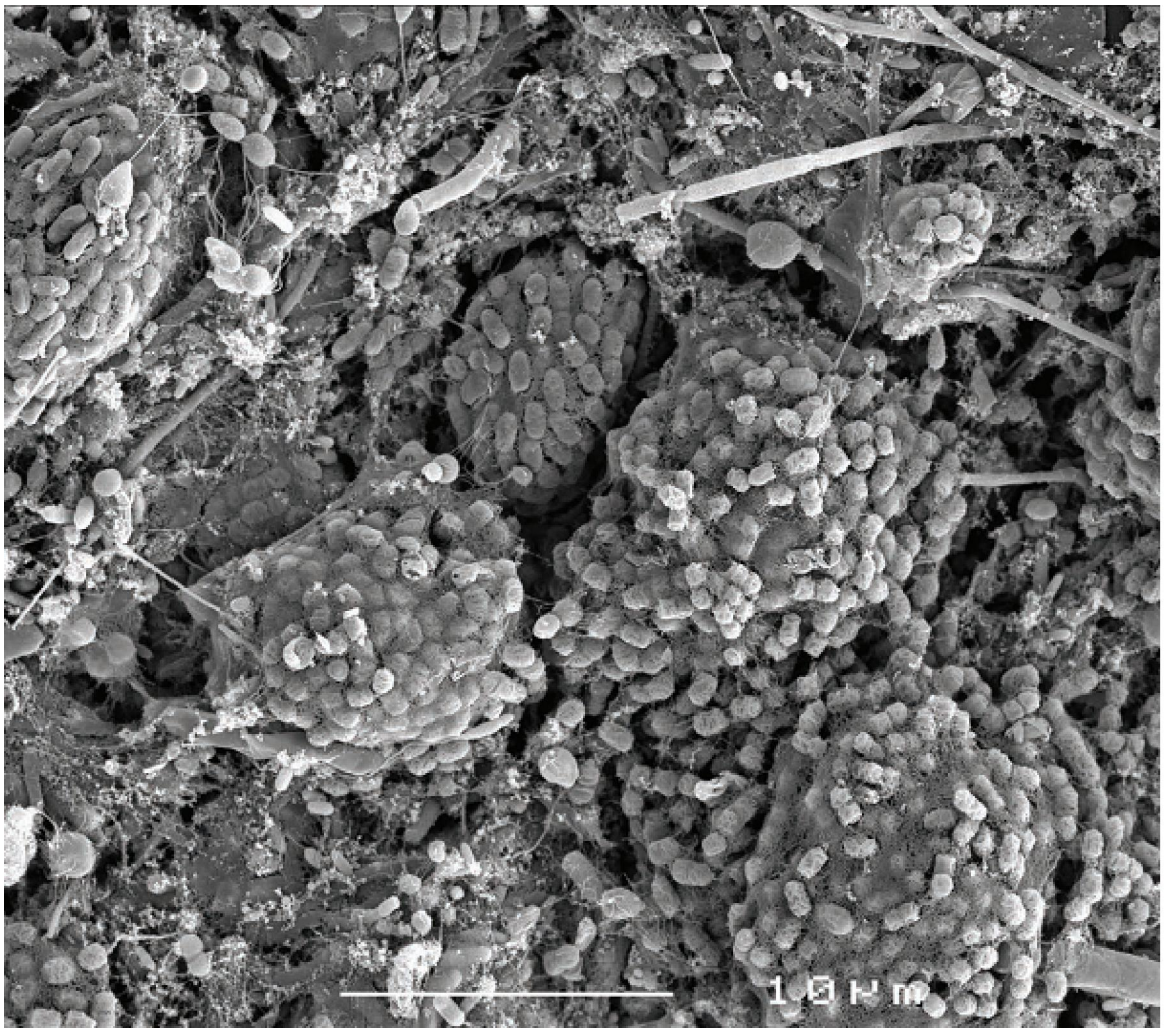


Chapter 1:

Introduction

1.1 Biofilm-An Overview

Microorganisms have the natural tendency to attach to biotic and abiotic surfaces, to multiply and to embed themselves in a three-dimensional gelatinous slimy matrix of self-produced extracellular polymeric substances (EPS) comprising of polysaccharides, proteins, DNA and other substances. This structured consortium of pure or mixed population of cells is called biofilm (Stewart *et al.*, 2008, Costerton *et al.*, 1995).

From the very beginning of microbiology as a research field, microorganisms have long been considered to be freely suspended cells and have been described on the basis of their morphological and physiological properties and growth characteristics in nutritionally rich culture media. Although first documented in 1943 by Zobell in which bacteria were found to be associated with surfaces, it was thought that these were isolated examples (Costerton, 2004). In the late 1970s, studies were conducted which showed that the vast majority of bacteria in oligotrophic environments were surface-associated (Costerton, 2004).

Today biofilm is considered as the most prevalent mode of growth of microorganisms. They are universally present on a broad variety of systems such as rhizospheres of plants and bodies of animals and humans, and of substrata as minerals, metals, stones, medical implants, paper, plastic, engineered and industrial systems, food processing equipments and artworks and also in extreme environments such as acid mine drainages, hot springs, frozen glaciers, space stations and highly irradiated areas of nuclear power plants (Costerton *et al.*, 1987; Flemming, 2002; Edwards *et al.*, 2000; Decho, 2000; Satpathy,

1999, Paerl *et al.*, 1998 and Koenig, 1997). It is thought that more than 99% of all microorganisms on Earth are living in such aggregates (Costerton *et al.*, 1987).

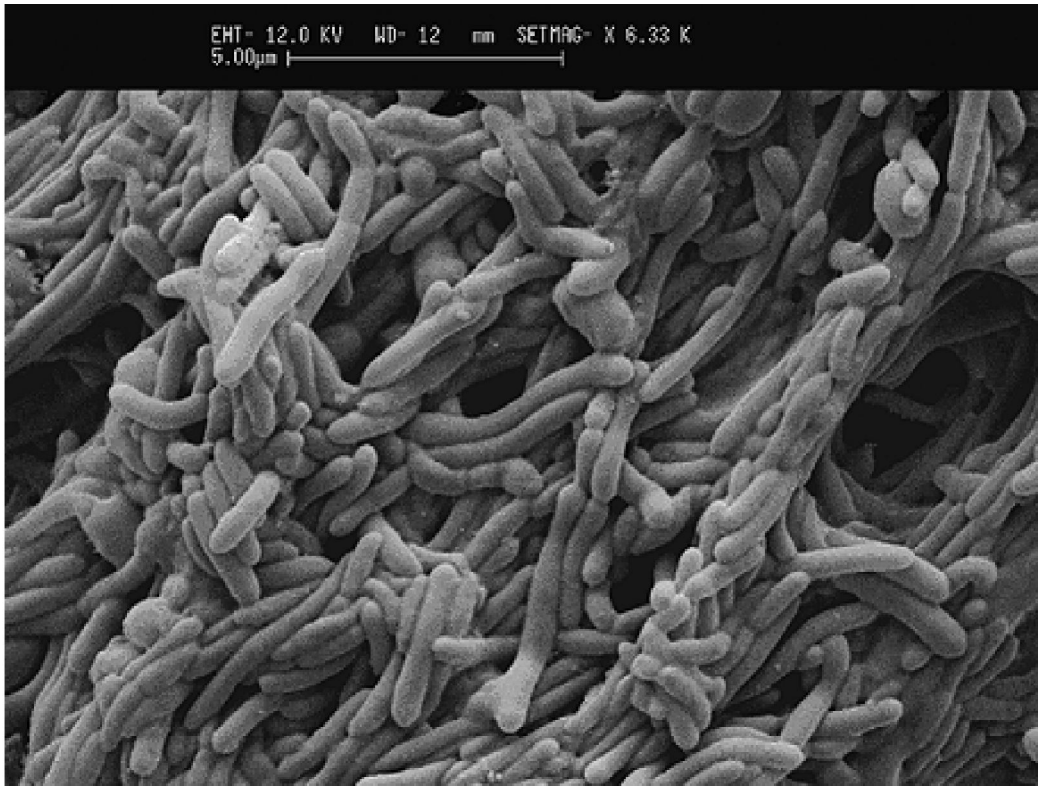


Figure 1.1: Scanning electron microscopy photomicrograph of a 6 days old *B. cereus* biofilm formed on a stainless steel surface. × 6330 magnification; bar = 5 µm.

1.2 The Biofilm Matrix

The biofilm matrix is a spatially well-organized biological structure that develops and persists at solid surfaces or at phase interfaces in aqueous environments (Ridgway et. al., 1996, Costerton et. al., 1995 and Donald, 2002). Depending on the environmental conditions, biofilms may be formed by either single or multiple microbial species. Under natural conditions, the monospecies biofilms are rare (Donald, 2002 and Tolker-Nielsen et. al., 2000). However, in most cases, a variety of microorganisms (e.g., bacteria, archaea, algae, fungi and protozoa) can result in multispecies consortia from an association between the metabolically cooperative organisms (Davey et. al., 2000).

The structure of the biofilm matrix may vary depending on the microbial cells present, their physiological status, prevailing physical conditions and above all on the available nutrients. The basic structural unit of each biofilm is the microcolony (Costerton, 1999) – an EPS matrix-encased communities of microbial cells that may include cells of one or of many species. (Sutherland, 2000). Under certain conditions, microcolonies can develop into a macroscopic structure several millimeters or centimeters in thickness and can cover large surface areas. Such macroscopic structure can be a single-cell layered, more or less confluent aggregate with a high degree of patchiness, low cell numbers and limited presence of polymeric compounds or be a multilayered, highly-organized, three-dimensional formation with non-uniform, mushroom-shaped or finger-like columns surrounded by fluid-filled channels and pores, multiple microbial species and different polymer compositions, different densities of active cells, etc. (Costerton et. al., 1995, Sutherland, 2000 and Stoodley et. al.,2002).

The major biofilm matrix components are microbial cells, polysaccharides and water, together with excreted cellular products. Approximately 97% of the biofilm matrix is either water, which is bound to the capsules of microbial cells, or solvent (Sutherland, 2001). Microbial cells constitute less than 10% of the dry mass in a biofilm (Flemming and Wingender, 2010). Apart from water and microbial cells, the extracellular matrix include a complex of secreted polymers, absorbed nutrients and metabolites, products from cell lysis and even particulate material and detritus from the immediate surrounding environment (Sutherland, 2001). In addition to peptidoglycan, lipids, phospholipids and other cell components, all major classes of macromolecules – proteins, polysaccharides, DNA and RNA – can be found within a biofilm matrix.

The three dimensional structure created by the microorganisms and the extracellular matrix provides mechanical support and allows the creation of a special environment where other components, such as enzymes and nutrients are maintained. The extracellular matrix components can themselves serve as nutrient source when degraded by enzymes. The matrix provides an important advantage for the microorganisms, as it protects from desiccation, oxidization, biocides, antibiotics, metallic cations and ultraviolet radiation (Branda *et al.*, 2005, Flemming and Wingender, 2010).

1.3 Stages of Biofilm Development

The exact molecular mechanisms for biofilm development may differ from organism to organism. However, the sequential five stages appear to be conserved among a wide range of microbes (Figure: 1.2) which include attachment of free-floating planktonic bacterial cells to surface, the growth and aggregation of cells into microcolonies followed by growth into mature, structurally complex biofilm, and finally dispersal of detached bacterial cells into the surrounding environment.

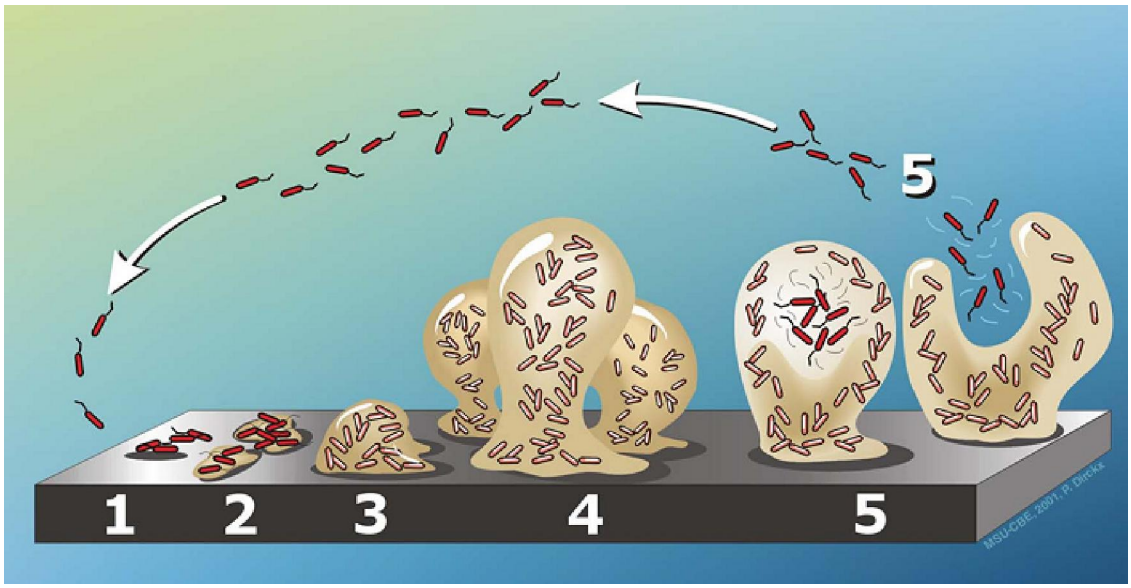


Figure 1.2. Developmental stages of biofilm formation (Adapted from *Annual Review of Microbiology*, vol. 56, 2002).

A. Reversible Attachment

The first step in biofilm development is surface colonization through reversible attachment. It is believed that two factors are required for initial reversible attachment to occur; first, conditioning of the target surface, and second, transport of bacteria to the surface.

The surface is conditioned with proteins, glycoproteins and organic nutrients, resulting in a nutritionally rich zone that is metabolically favorable for cells (Marshall *et al.*, 1984; Beveridge *et al.*, 1997). Planktonic bacteria are then transported to close proximity of the conditioned surface by either random (*e.g.* sedimentation and liquid flow) or in a directed fashion (*e.g.* chemotaxis and active motility (Quirynen *et al.*, 2000). This step is facilitated by the existence and strength of electrostatic forces, hydrophobic interactions and van der Waals forces depending on the proximity of the organism to the attachment surface (van Loosdrecht *et al.*, 1990).

B. Irreversible Attachment

The second step in bacterial biofilm development is irreversible attachment, during which production of bacterial exopolysaccharides (EPS) results in more stable attachment by forming organic bridges between the cells and substratum (Notermans *et al.*, 1991). The presence of flagella, pili and/or curli is also responsible for the transition from reversible to irreversible attachment process.

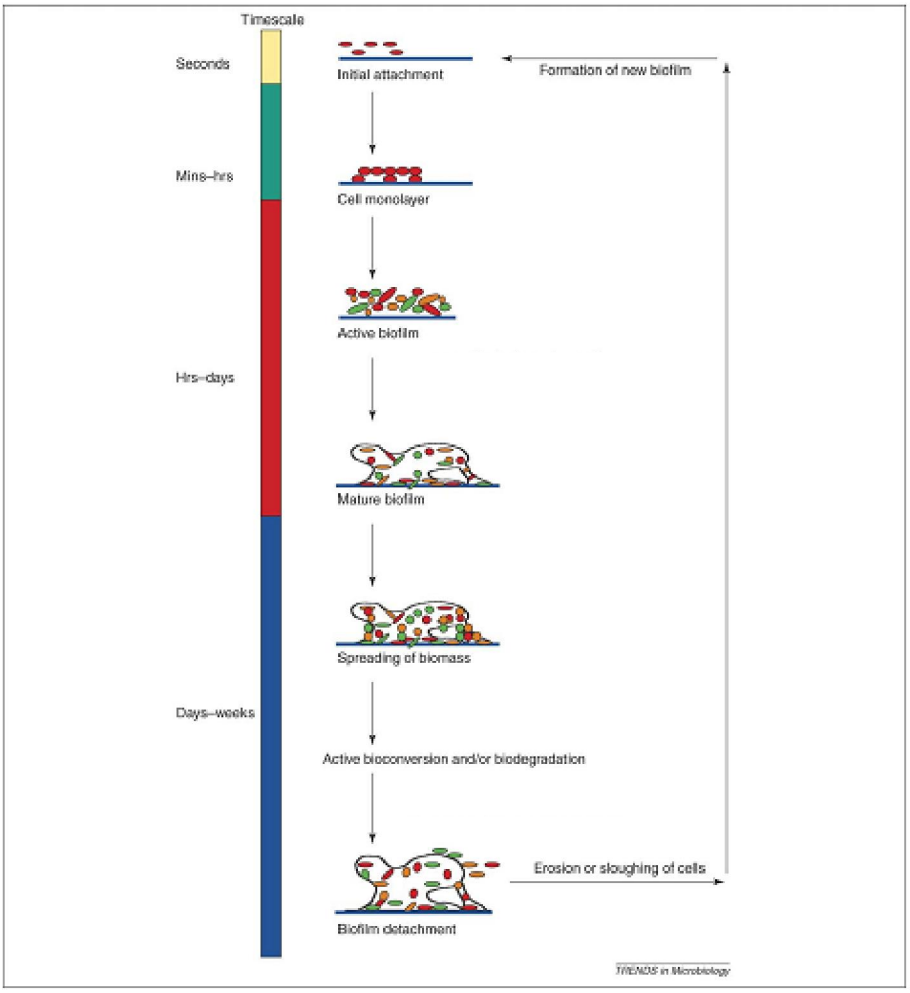


Figure 1.3: Diagrammatic representation of cyclic steps involved in the formation of an active biofilm (Adapted from Singh *et al.* (2006), *Trends in Microbiology*, 14:391).

C. Formation of Microcolonies

Once the bacteria have irreversibly attached to surfaces, binary division of irreversibly attached cells causes the daughter cells to spread outward and upward from the attachment point to form microcolonies or cell clusters (Tolker-Nielsen *et al.*, 2000). The microcolony is actually the basic structural unit of the biofilm. Depending on the species involved, the

microcolony may be composed of 10–25% cells and 75–90% EPS matrix, and the matrix material often appears to be most dense in the area closest to the core of the microcolony.

D. Biofilm Maturation

The matured three dimensional mushroom- or pillar-like structures of biofilms are developed from microcolonies. Also during this stage, extracellular polymeric substances continue to be produced. A mature biofilm is usually composed of a complex architecture containing live and dead cells plus a substantial amount of extracellular material. Water channels and pores are also formed, in which bacteria can develop specific patterns of growth and a different physiology and metabolism from planktonic cells.

E. Detachment

Bacterial participation in biofilm populations are limited by the availability of nutrients in the environment and accumulation of toxic by-products and other factors, including pH, oxygen perfusion, carbon source availability and osmolarity (Davies *et al.*, 1998; O'Toole *et al.*, 2000). At some point, bacteria will detach from the biofilm and once again transform into their planktonic form. Several mechanisms for biofilm dissolution and consequently, cell dispersal have been proposed. Loss of EPS or the productions of enzymes such as polysaccharide lyase and alginate lyase have been reported to play a role in biofilm dissolution in several organisms (Sutherland, 2000; Kaplan *et al.*, 2003).

1.4 Factors Affecting Biofilm Formation

There are numerous factors (Table 1.1) that attribute to the attachment of microorganisms to surfaces and the subsequent biofilm formation. Nature of the attachment structures, properties of the cell surface, particularly the presence of extracellular appendages such as flagella and fimbriae, the interactions involved in cell–cell communication (quorum sensing) and EPS production are important for biofilm formation and development (Allison, 2003; Davies *et al.*, 1998; Donlan, 2002; Parsek and Greenberg, 2005; Sauer and Camper, 2001).

Table 1.1: Variables important in cell attachment, biofilm formation and development (based on Donlan, 2002).

Adhesion surface	Bulk fluid	Cell
Texture or roughness	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	pH	Extracellular appendages
Surface chemistry	Temperature	Extracellular polymeric substances
Charge	Cations	Signalling molecules
Conditioning film	Presence of antimicrobial products	
	Nutrient availability	

A. Specialized Attachment Structures

In general, the attachment of microorganisms to a surface is a very complex process with many variables affecting the outcome. Microorganisms tend to attach readily on surfaces that are rougher, more hydrophobic and preconditioned with organic materials (Donlan, 2002).

According to Characklis *et al.* (1973), the extent of microbial colonization increases as the surface roughness increases. This is because shear forces are diminished, and surface area is higher on rougher surfaces. Hydrophobic surfaces play a critical role in attachment as most investigators have found that microorganisms attach more rapidly to hydrophobic, non-polar surfaces such as Teflon and other plastics than to hydrophilic materials such as glass or metals (Fletcher *et al.*, 1979; Pringle *et al.*, 1983 and Bendinger *et al.*, 1993). Allison *et al.* (2003) noted that surfaces cannot be colonized by biofilms unless they have been exposed to organic material from the surrounding environment.

However, apart from physicochemical properties of the surface, an increase in flow velocity or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels (Simoes *et al.*, 2007; Stoodley *et al.*, 1999; Vieira *et al.*, 1993).

B. Properties of the Cell

Cell surface hydrophobicity, presence of extracellular filamentous appendages and production of extracellular polymeric substances (EPS) may influence the rate and the extent of attachment of microbial cells.

Hydrophobicity of the cell surface is important because hydrophobic interactions tend to increase with an increasing non-polar nature of one or both surfaces involved (i.e., the microbial cell and the adhesion surface) (Donlan, 2002). Most bacteria are negatively charged but still contain hydrophobic surface compounds such as flagella and fimbriae. Flagella, when present, help bacteria in transportation and initial cell-surface interactions during the initial stages of biofilm development (Sauer and Camper, 2001). Flagella-mediated motility is believed to overcome repulsive forces at the surface of the substratum and, as a consequence, a monolayer of cells forms on the adhesion surface (Daniels *et al.*, 2004). On the other hand, fimbriae play a role in cell surface hydrophobicity and attachment, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and substratum (Corpe, 1980).

In addition to hydrophobic interactions, EPS are responsible for attachment to hydrophilic materials. One of the most important functions of EPS is to bind cells and other particulate materials together (cohesion) and to the surface (adhesion) (Allison, 2003; Characklis and Wilderer, 1989; Sutherland, 2001). The EPS matrix of a biofilm structure determines the mechanical stability of biofilms, mediated by non-covalent interactions either directly

between the polysaccharide chains or indirectly via multivalent cation bridges (Flemming 1996; Allison, 2003).

C. Cell Communication (Quorum Sensing)

Cell-to-cell communication is essential for biofilm formation. The development of biofilms on surfaces is mediated by a density dependent chemical signal released by bacterial cells densely packed within an EPS matrix. Microorganisms can use quorum sensing to coordinate their communal behavior such as biofilm formation, motility and production of EPS (Xiong and Liu, 2010).

Quorum systems make use of a transcriptional activator protein that acts in concert with a small autoinducers (AI) signaling molecule to stimulate expression of target genes (de Kievit *et al.*, 2001). Oligopeptides and N-acylhomoserine lactones (AHL) are major AI molecules involved in intra-specific communication in Gram-positive and Gram-negative bacteria, respectively, whereas boronated diester molecules (AI-2) are involved in inter-specific communication among both Gram-positive and Gram-negative bacteria (Eberhard *et al.*, 1981; Fuqua and Greenberg, 2002; Parsek and Greenberg, 2005).

Quorum sensing signals can also control biofilm detachment by the accumulation of the signal molecules (excreted by bacteria) to a threshold concentration which will eventually trigger the dispersion of the biofilm (Daniels *et al.*, 2004).

1.5 Impacts of Biofilm Formation

The consequences of bacterial adhesion to surfaces and subsequent biofilm formation can be beneficial or deleterious depending on the situation. If occurred "at the wrong place" and "at the wrong times", biofilm formation is often considered as a harmful event. However, there are also much positive ecological and industrial significance of biofilms.

A. Positive Impacts

From microbial prospective, biofilm formation offers advantages by providing protection from competing microorganisms and environmental factors such as host defense mechanisms (phagocytosis and other immune responses) and from potentially toxic substances in the environment, such as biocides, disinfectants or antibiotics.

From ecological point of view, biofilms are conducting a number of biological processes, such as photosynthesis, processing and uptake of organic matter, removal of many potentially harmful environmental pollutants and cycling of carbon, hydrogen, nitrogen, sulphur, phosphorus and many metals (Edwards *et al.*, 2000; Paerl and Pinckney, 1996).

Industrial applications of biofilm include biomineralization and bioremediation (McLean and Beveridge, 1990; Ebihara and Bishop, 1999), water and wastewater treatment (Nicolella *et al.*, 2000; Massol-Deya *et al.*, 1995), biofuel production (Wang and Chen, 2009) and even generation of electricity in microbial fuel cells (Rabaey *et al.*, 2007).

In addition, biofilms of commensal bacteria in the human gastrointestinal tract are important for maintaing good health, as alterations in the gut microbial community can lead to indigestion and other diseases (Sekirov *et al.*, 2010).

B. Negative Impacts

Biofilm formation contributes to a range of costly problems in daily life. These detrimental effects can range in severity from being a mere nuisance to being life threatening.

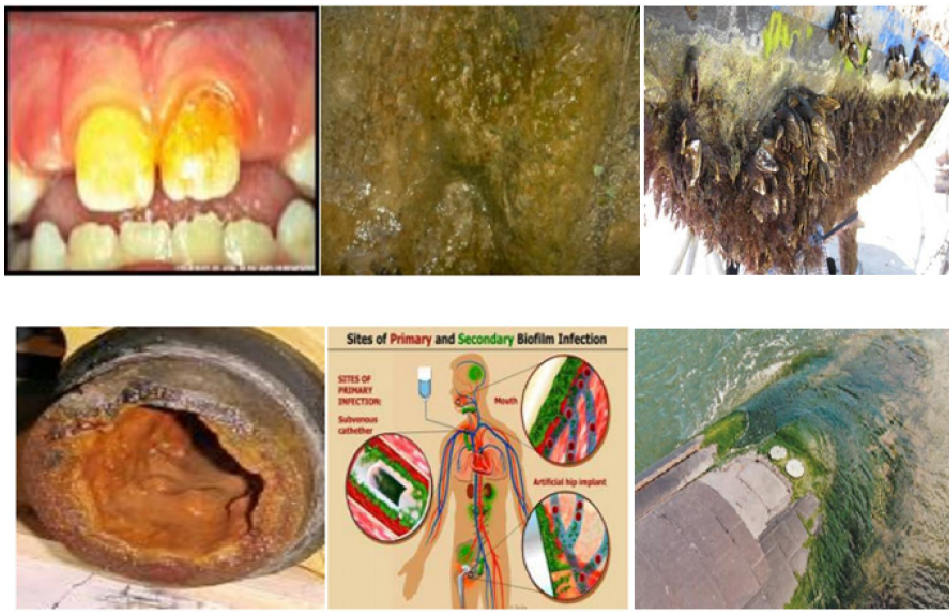


Figure 1.4: Biofilms found on teeth (top left), rocks and pebbles (top middle), bottom of vessel (top right), inside of pipes (bottom left), human biofilm infection sites (bottom middle), and above sea shore (bottom right) (Source: www.poolcare.net).

Table 1.2: Detrimental effects of biofilm processes (according to Srinivasan *et al.*, 1995).

System	Effects
Cooling water towers and heat exchangers	Energy losses due to increased fluid frictional and heat transfer resistances
Drinking water distribution	Increased suspended solids; coliform contamination
Secondary oil recovery	Plugging of water injection wells corrosion
Process equipment	Material corrosion or biodeterioration
Food processing	Contamination
Metalworking	Degradation of metal working fluid
Paper manufacture	Degradation of product quality
Dental plaque	Caries; periodontal disease
Medical implants, catheters	Persistent infections
Ship hulls	Increased frictional drag
Reverse osmosis membranes	Reduced permeability; material degradation
Clean surfaces (health care, consumer)	Health risks; cosmetic degradation
Swimming pools	Health risks; cosmetic degradation

Up to 80% of human bacterial infections are usually caused by biofilm forming bacteria (Costerton *et al.*, 1999). Biofilms are responsible for some nosocomial infections and chronic diseases. Immunocompromised and cystic fibrosis patients are prone to *Pseudomonas aeruginosa*, a common biofilm-forming opportunistic pathogen, which can lead to impairment of the lung (Smith and Hunter, 2008). Other diseases frequently attributed by biofilms on host tissues include colitis, vaginitis, urinary tract infections, endocarditis, conjunctivitis and otitis media (Donlan and Costerton, 2002; Ehrlich *et al.*, 2004). Moreover, biofilm formation is a common cause of oral diseases such as dental

caries, periodontitis, and denture stomatitis (Donlan and Costerton, 2002). Furthermore, virtually all indwelling medical devices such as prosthetic heart valves, orthopedic devices, contact lenses and urinary catheters are prone to colonization by biofilm forming pathogenic bacteria and can serve as a source for recurrent infections (Donlan and Costerton, 2002).

Biofilms also have significant contribution in food-borne illness. Biofilms of *E. coli*, *Listeria monocytogenes*, and *Campylobacter jejuni* remain a significant safety challenge within the food industry (Wood, 2009; Gandhi and Chikindas, 2007; Murphy *et al.*, 2006). The presence of any of these pathogens in dairy, poultry or meat industry can cause cross-contamination of processing equipment, leading to widespread production and post-production contamination that can reach the consumer (Kumar and Anand, 1998).

Biofilms often cause physical damages, chemical alterations, loss of functionality and aesthetic changes to a range of industrial processes. Biofilm-associated microbial activity may cause fouling, corrosion and/or blockages in condenser tubes, sensors, water and wastewater circuits, membrane modules, heat exchange tubes and even on ship hulls (Bott, 1995; Chang *et al.*, 2002; Emde *et al.*, 1992), leading to significant economic losses and environmental damages every year. Moreover, biofilms in cooling towers, shower curtains, water reservoirs and distribution pipelines pose serious microbial threat (e.g. *Legionella pneumophila* and *E. coli*) to human health (Flemming, 2002; Juhna *et al.*, 2007).

1.6 Approaches for Biofilm Control

A. Conventional approaches to treat biofilm

The control of the deleterious biofilm is today a great challenge and is greatly dependent on the type and nature of the contaminating residue materials and the microorganisms to be removed from the surfaces. From the very beginning of biofilm discovery, several conventional methods have been used to prevent and destroy biofilms. They include:

I. Physical and/or mechanical removal

II. Chemical removal

III. Use of antimicrobials

I. Physical and/or mechanical removal

Scrubbing, heating, sonication, freezing and use of ultrasound and high pressure have been used to eradicate biofilms from surfaces. Mechanical approaches have long been the most effective ways of removing established biofilms associated with surfaces without having much bactericidal effect. However, spreading surviving microbes via aerosol by many mechanical actions (scrubbing, high pressure etc.) make physical approach impractical as bacteria can redeposit at other locations and given time, water and nutrients can again give rise to a new biofilm mode of growth. Moreover, the mechanical forces are sometimes destructive towards the treating material surface and can be very expensive. In addition, most of the physical and/or mechanical removal approaches are limited to small areas.

II. Chemical removal

Chemical approach for biofilm removal consists mainly in the application of biocides such as ozone, hypochlorite (bleach), hypobromite, chloramine, chlorine dioxide, and hydrogen peroxide. Many biocidal paints containing organotin (tributylin) and copper compounds have been developed to prevent biofilm formation that lead to fouling of surfaces by macroscopic organisms in marine ecosystems. Although biocides have had success in destroying or preventing biofilm formation, the use of traditional biocides has several disadvantages. Biocides are generally non specific in their action and often become potentially harmful both for human and the environment. Constant exposure, misuse and low biodegradability make biocide unfriendly and may lead to loss of biodiversity and pollution problems in different environments as well as chronic health impairments in humans such as cancer, birth defects, reproductive problems and sensitization. Moreover, biocides become ineffective over a period of time due to their loss of activity and low diffusion ability into the EPS of biofilm which in turn can enhance natural resistance towards biocides among microbes within the biofilms. Recent studies revealed that biocides such as triclosan, benzalkonium chloride and chlorhexidine gluconate, which are commonly used on surfaces, are completely ineffective in removing biofilm (Smith and Hunter, 2008).

III. Use of antimicrobials

Several attempts have been made to avoid biofilm formation through the incorporation of antimicrobial agents into surface materials or by coating surfaces with antimicrobials (Meyer, 2003). Although some recently discovered antibiotics such as Azythromycin and Clarithromycin have been found to possess anti-biofilm effect to some extent at their minimum inhibitory concentration (MIC), they were found to have no effect against established biofilms (Gillis and Iglewski, 2004; Carter *et al.*, 2004). However, inherent diffusion limitations, inability to effect non-proliferating regions of biofilm and high level of differentiation in the local environment within the biofilm may render the use of even high concentrations of antimicrobials ineffective in killing cells within the biofilm and furthermore can result in enhanced levels of biofilms' future resistance properties through selection of resistant mutants (Stewart *et al.*, 2004). Stewart and Costerton in 2001 noted that biofilms can be as much as a thousand times more resistant to antimicrobials than planktonic cells. Use of antimicrobials does not often result in efficient or successful removal of biofilm, which in turn may eventually release planktonic cells that can cause a systemic infection.

Among other notable conventional approaches, synergistic use of enzymes (such as proteases and polysaccharide hydrolyzing enzymes) with detergents boost disinfectant efficacy while controlling biofilm. However, the specific mode of action makes this method impractical against different types of biofilms.

B. Novel approaches for biofilm treatment

Presently there is no known traditional practice that is able to successfully prevent or control the formation of unwanted biofilms without causing deleterious side effects. In addition, environmental concerns and emergence of resistant cells due to traditional killing of biofilm forming bacteria do not present safe and long term solution to the challenge imposed by biofilm. Therefore, novel anti-biofilm strategies need to be devised, which should be more effective, economic and above all pose negligible risk to human health and the environment. For the last few years, several novel approaches able to control biofilm development have been proposed as alteration to the traditionally active substances (Figure 1.5).

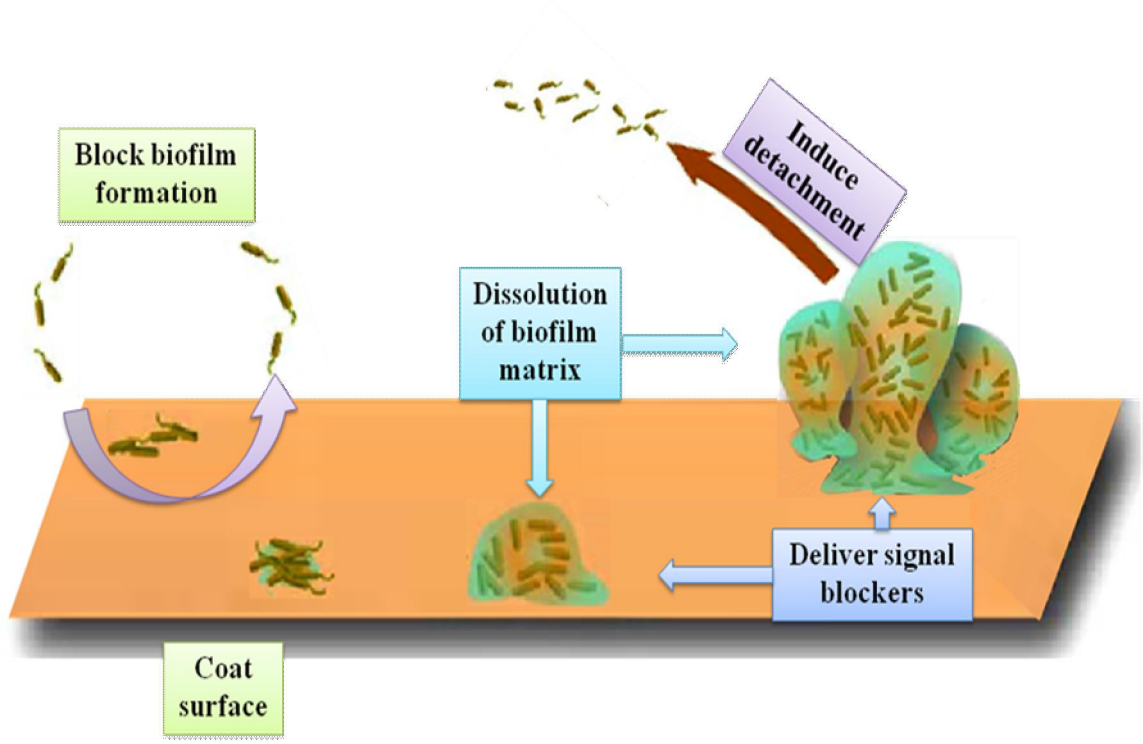


Figure 1.5: Novel strategies to prevent biofilm formation (Adapted from Rickard *et al.* (2003) *Trends in Microbiology*, 11:94)

Once a biofilm is established, resistance properties of life within biofilm make the eradication of biofilm difficult to accomplish. The prevention of the onset of biofilm formation would therefore be a more attractive control strategy. Most of the recent researches carried out so far mainly focuses on blocking biofilm formation before bacteria attach firmly to surfaces. Blocking the initial attachment to surfaces was found to prevent initial biofilm colonization as well as the subsequent mature biofilm formation.

A number of approaches have targeted on preventing the initial adhesion of bacteria to a surface or lowering the force of adhesion between bacteria and a surface to facilitate removal. Several anti-adhesion strategies have been proposed, including the development of receptor blocks, pre-conditioning of the surfaces with biosurfactants, polysaccharides or other bioactive compounds or alteration of the physicochemical properties of the outermost layer of the biofilm forming cells for interfering cell-to-surface and/or cell-to-cell communication (Bavington, 2005; Ofek *et al.*, 2003; Valle *et al.*, 2006). Physical properties such as surface energy (hydrophobicity or hydrophilicity), surface roughness, steric hindrance of surface groups, and electrostatic interactions have been found to display critical roles in blocking the onset of initial attachment of bacteria to surfaces. Receptor blocks may alter ligand-receptor binding, preventing the bacterial recognition of a surface (Ofek *et al.*, 2003).

Surface pre-conditioned with non-ionic and anionic surfactants were successfully evaluated in preventing the adhesion of *Pseudomonas aeruginosa* to stainless steel and glass surfaces (Cloete and Jacobs, 2001). In other studies, bacteria such as *Lactobacillus*

lactis and *Bacillus subtilis* were found to be able to synthesize and excrete biosurfactants with anti-adhesive properties without affecting cell growth (Rodrigues *et al.*, 2004; Mireles *et al.*, 2001). A more recent study revealed that a uropathogenic strain of *E. coli* expressing group II capsules release a soluble polysaccharide into the surrounding environment that prevents biofilm formation of *E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Staphylococcus epidermis*, and *Enterococcus faecalis* without affecting their planktonic growth rate (Valle *et al.*, 2006). The polysaccharide capsule believed to exert its anti-adhesion property by electrostatically modifying surfaces and reducing cell aggregation.

Other notable novel biofilm control strategies include the use of quorum sensing inhibitors and disassembling the existing biofilms. The discovery that wide spectrums of bacteria use quorum sensing to perform biofilm formation and differentiation makes it an attractive target for biofilm control. Janssens *et al.* (2008) and Brackman *et al.* (2009) used quorum sensing inhibitors to reduce biofilm formation. Both the research groups noticed biofilm reduction without significantly reducing the planktonic cell population through targeting quorum sensing systems. Disassembling of existing biofilms was also recently achieved by four D-amino acids namely D-tyrosine, D-leucine, D-tryptophan, and D-methionine (Kolodkin-Gal *et al.*, 2010). Moreover, *S. aureus* and *P. aeruginosa* cultures were unable to form biofilms in the presence of D-tyrosine and the D-amino acid mixture (D-tyrosine, D-leucine, D-tryptophan, and D-methionine).

1.7 Natural Anti-biofilm Compounds

Bacteria in a biofilm can affect the growth of other bacteria in the same biofilm (Burgess *et al.* 1999) by producing growth inhibitory or anti-biofilm compounds. For example, the presence of ‘‘resident’’ bacterial strains on particles either increases or decreases the colonisation rate of ‘‘newcomer’’ strains (Grossart *et al.* 2003). The marine bacterium *Alteromonas* sp. produces 2-n-phenyl-4-quinolinol that alters the composition of the bacterial community developed on particles (Long *et al.* 2003).

Natural anti-biofilm compounds isolated so far from microorganisms comprises mainly secondary metabolites ranging from furanone to exopolysaccharide. The very first anti-biofilm compound isolated was a quorum sensing antagonist (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl- 2(*5H*)-furanone (furanone) from the marine red alga *Delisea pulchra* that inhibits biofilm formation in *E. coli* and *Bacillus subtilis* without inhibiting its growth (Ren *et al.*, 2001; Ren *et al.*, 2002).

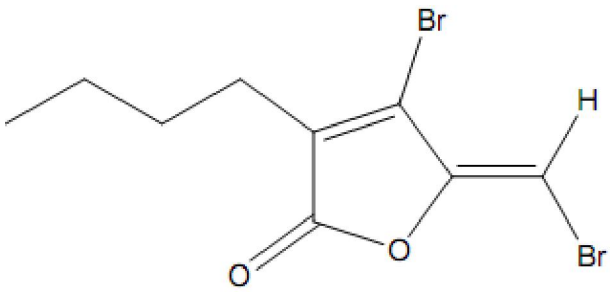


Figure 1.6: Brominated furanone, (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone.

Ursolic acid from *Diospyros dendo* was shown to inhibit the formation of *P. aeruginosa*, *Vibrio harveyi*, and *E. coli* biofilms, and to disperse established biofilms of *E. coli* (Ren *et*

al., 2005). Other plant extract ursene triterpene compounds extracted from *Diospyros dendo* inhibit biofilm formation in *P. aeruginosa* by 32% to 62% (Hu *et al.*, 2006). A mixture of three compounds (betonicine, α-D-galactopyranosyl-(1→2)-glycerol (floridoside), and isethionic acid) purified from the red alga *Ahnfeltiopsis flabelliformis*, was reported to have an inhibition activity on bacterial quorum sensing mechanism mediated by N-octanoyl-DL-homoserine lactone (Kim *et al.* 2007).

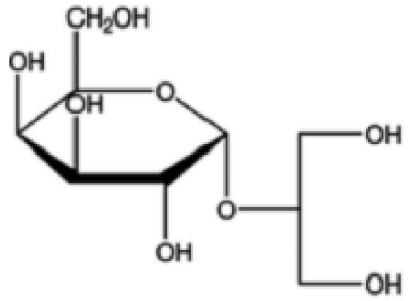


Figure 1.7: α-D-galactopyranosyl-(1→2)-glycerol (floridoside)

The metabolites of a marine actinomycete strain A66 inhibit biofilm formation by *Vibrio* in marine ecosystem (You *et al.* 2007). Extracts from coral associated *Bacillus horikoshii* (Thenmozhi *et. al.* 2009) and actinomycetes (Nithyanand *et. al.* 2010) inhibit biofilm formation of *Streptococcus pyogenes*. Most recently, some polysaccharides secreted from marine and non marine organisms were found to possess the ability to negatively regulate biofilm formation. For example, exoplysaccharides from *E. coli* (group II capsular polysaccharide), *V. vulnificus* (capsular polysaccharide), *P. aeruginosa* (mainly extracellular polysaccharide), marine bacterium *Vibrio* sp.QY101 (exopolysaccharide) and *Kingella kingae* (exoplysaccharide) display selective or broad spectrum anti-biofilm activity (Valle *et al.*, 2006; Joseph and Wright, 2004; Qin *et al.*, 2009; Jiang *et al.*, 2011; Meriem *et al.*, 2011).

1.8 Marine Sponge-Associated Bacteria as a Source for Anti-biofilm Compounds

In marine environments, all unprotected artificial and natural surfaces quickly become colonized by both micro- and macro-organisms, a process known as biofouling (Wahl, 1989). Biofouling is a global phenomena and involves i) adsorption of dissolved organic molecules to a newly submerged surface, ii) colonization of the surface by bacteria (biofilm), iii) colonization by microscopic eukaryotes (e.g. diatoms, fungi, and other heterotrophic eukaryotes) and iv) settlement and subsequent growth of invertebrate larvae and algal spores (macrofouling). As the formation of biofilms on newly submerged substrata attracts colonization by invertebrates, the establishment of microbial biofilms or symbiotic associations of bacteria with marine invertebrates appear to play the most crucial role in biofouling.

To combat against this biofouling, marine organisms, from bacteria to invertebrates, employ several strategies to maintain their body free from potential invaders, predators or other competitors. Organisms alone (directly) or in symbiotic association (indirectly) often secrete potential antibacterial, anti-biofilm and anti-fouling secondary metabolites in the surrounding environment to defend themselves. For example, marine sponges secrete some secondary metabolites that directly inhibit unwanted bacterial association on their surface as well as other biofouling organisms to settle down on their surface (Figure 1.8).

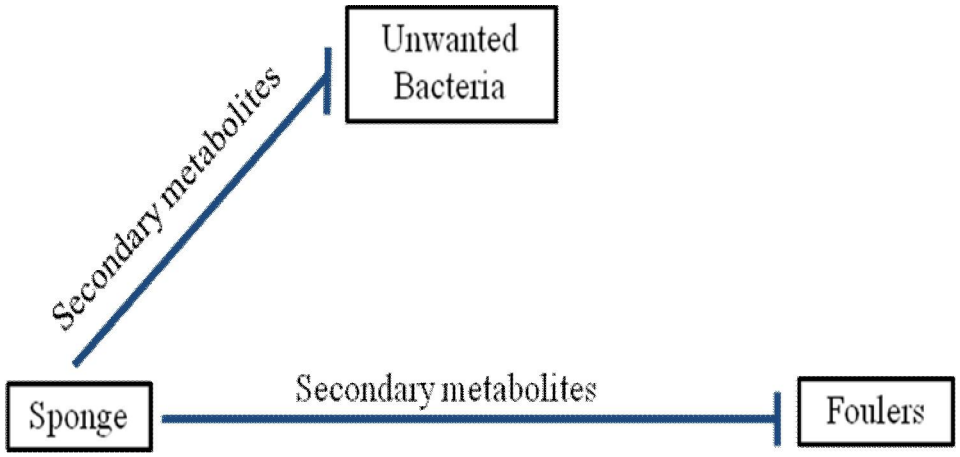


Figure 1.8: Direct way for defending against biofouling in marine environment by sponge.

On the contrary, sponges can also chemically attract desired bacterial community to grow on their surface as biofilm. This associated desired bacterial community in return can inhibit or interfere the subsequent settlement of unwanted bacteria or macrofoulers on the surface of the host sponge (Figure 1.9).

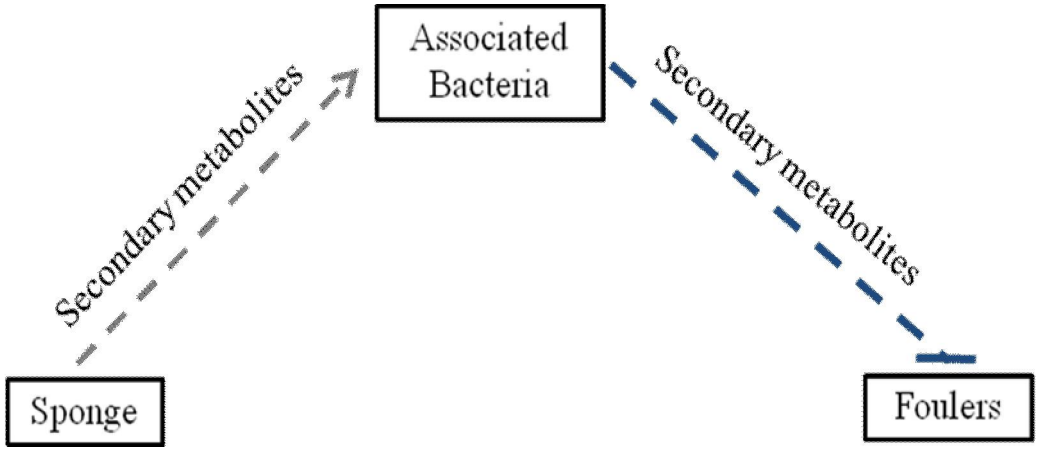


Figure 1.9: Indirect way for defending against biofouling in marine environment by sponge.

Until last decade, the true source of the bioactive compounds from sponges, corals and other marine invertebrates remained unclear. Recent studies revealed that several potentially bioactive compounds identified in sponges have striking similarities to metabolites derived from their associated microorganisms (Proksch *et al.*, 2002; Thiel and Imhoff, 2003). For instance, an anti-bacterial peptide that was originally isolated from the sponge *Hyatella* sp. is synthesised by an associated *Vibrio* sp. (Oclarit *et al.*, 1994). In addition, bioactive metabolites isolated from the sponge *Theonella swinhoei* by Bewley *et al.* (1996) could be produced by microbial symbionts rather than by the sponge itself (Thakur and Anil, 2000). Now it is well established that many of the bioactive compounds previously thought to be of sponge origin are in fact biosynthesized through microorganisms associated with the sponges or are produced entirely by these organisms (Fenical and Jensen, 1994; Fusetani and Clare, 2006).

It is now well established that sponges harbor taxonomically diverse microorganisms on their surfaces as well as within their bodies (Simpson, 1984). As a consequence, sponge-associated marine bacteria are becoming emerging potential sources for novel biosurfactants (Gandhimathi *et al.*, 2009; Kiran *et al.*, 2009) as well as antibacterial and antilarval-settlement (Das *et al.*, 2009). In fact, for the last decade, much research has been done focusing mainly on marine bacteria associated with the marine sponges (Blunt *et al.* 2006). Therefore, search for novel secondary metabolites as anti-biofilm agent from sponge-associated bacteria could be a promising approach.

Chapter 2:

Aims of the work

Due to huge negative impacts on public health, food industry, sewage and water system, marine transportation and above all the increased resistance of cells within biofilms, it is now a major challenge for human being to device a more reliable, environmentally compatible approach to combat against biofilm. Although numerous approaches have been developed in the last few years, much is still to be focused on different natural phenomena to find the possible solutions.

For every natural problem, there is always a natural solution. In marine environment, as already described in the previous chapter, sponges defend themselves directly against biofouling by secreting secondary metabolites. In addition, selective bacterial symbiotic association with marine sponge or biofilm community on its surface play a significant role in attracting or inhibiting the subsequent stages of biofouling formation. The discovery of huge microbial diversity in marine sponges provides unprecedented research opportunities. The surfaces and internal spaces of marine sponges provide unique microhabitats where bacteria are regularly detected. These environments contain more nutrients than the seawater and sediments thus providing a rich source for the isolation of diverse bacterial species with diverse metabolic capabilities and potentials. In many cases, they may be considered as a possible source of anti-biofilm compounds. Although marine symbiotic microbes have been proved to be a promising source of antifouling and antimicrobial compounds, only a small number of marine sponge associated bacteria have been screened for their anti-biofilm properties so far, and only a very few compounds have been successfully pooled out from such sources.

There are a number of benefits determined by using sponge associated microorganisms as sources of anti-biofilm compounds. The first advantage is the relief of problems relating to commercial supply of the bioactive compounds. For the extraction of a compound from a marine sponge, large numbers of wild or rare species of sponge would have to be collected. This would lead to their possible extinction from the environment. In contrast, the sponge associated bacteria can be easily cultured, provided that adequate knowledge for the isolation and cultivation techniques is available. Another advantage is that microorganisms can produce compounds much more rapidly and in large amounts compared to sponges. Moreover, bacterial strains of the same species can produce different bioactive compounds under different culture conditions, therefore, increasing the potential number of useful compounds. Although there are numerous examples of compounds being extracted from marine sponge associated bacteria which have anti-microbial or anti-larval properties, there is very little information on anti-biofilm compounds from marine sponge associated microorganisms.

The above phenomena open up novel research alleys for the development of environmentally compatible natural products from marine sponge associated bacteria for anti-biofilm activities with which we can thoroughly control biofilm formation. The present research was initially focused on the compounds from a Mediterranean sponge, *Spongia officinalis*, for selective bacterial association and then isolation of secondary metabolites from the *Spongia officinalis*-associated bacteria for anti-biofilm activity against well studied reference strains such as *Escherichia coli* PHL628 and *Pseudomonas fluorescens*.

The main goals of the present research include:

1. Assessment of compounds from a marine sponge for any kind of inducing effect on the biofilm development by a well studied reference strain.
2. Screening and assessing bacteria from the same sponge for anti-biofilm compounds.
3. Purification of the secreted compounds displaying anti-biofilm activity.
4. Characterization of the anti-biofilm compound.
5. Preliminary characterization of the mechanism of action of the anti-biofilm compound.

Chapter 3:

Materials and Methods

Bacterial cultures used

Escherichia coli strain PHL628, used in this study, is a well known biofilm-forming K12 MG1655 derivative with an ompR234 mutation that causes it to over express curli and attach to surfaces. Another well studied biofilm producing strain used in several experiments of the present work is *Pseudomonas fluorescens*. In addition, a number of laboratory strains such as *Acinetobacter*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhimurium*, *Shigella sonneii*, *Listeria monocytogenes*, *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Bacillus pumilus* and *Bacillus subtilis* were used to assess the multispectrum anti-biofilm activity.

Effect of compounds from Spongia officinalis on E. coli PHL628 *biofilm*

13 purified compounds such as anhydrous furospongin-1, tetrahydrofurospongin-2, dihydrofurospongin-2, furospongin-1, furospongolide, furospongin-4, 12α-deoxoscalarin, 16-deacetoxy-12-epi-scalarafuranacetate and scalaradial from a orange-colored Mediterranean sponge, *Spongia officinalis*, were used to test the effect on biofilm formation by *E. coli* PHL628.

The method used for biofilm assay was a modified version of that described by Djordjevic *et al*. 2002. Overnight cultures of *E. coli* PHL628 strain grown at 37°C in $M63K_{10}$ broth (M63 broth with kanamycine, 10µg ml^{-1}), were refreshed in $M63K_{10}$ broth and incubated again at 37°C for 5 to 6 h. 200 µl of inoculum was introduced in the 96 well polystyrene microtiter plate with an initial turbidity at 600 nm of 0.05 in presence of different

concentrations of each purified compounds obtained from the marine sponge. The microtiter plate was then left at 30°C for 36 h in static condition.

To correlate biofilm formation with planktonic growth in each well, the planktonic cell fraction was transferred to a new microtiter plate and the OD_{570} was measured using a microtiter plate reader (*Multiscan Spectrum, Thermo Electron Corporation*). To assay the biofilm formation, the remaining medium in the incubated microtiter plate was removed and the wells were washed five times with sterile distilled water to remove loosely associated bacteria. Plates were air-dried for 45 min and each well was stained with 200µl of 1% crystal violet solution for 45 min. After staining, plates were washed with sterile distilled water five times. The quantitative analysis of biofilm production was performed by adding 200µl of ethanol-acetone solution (4:1) to de-stain the wells. The level (OD) of the crystal violet present in the de-staining solution was measured at 570 nm. Normalized biofilm was calculated by dividing the OD values of total biofilm by that of planktonic growth. Six replicate wells were made for each experimental parameter and each data point was averaged from these six.

Screening for bacterial strains from Spongia officinalis

The same sponge, *Spongia officinalis*, was used for the isolation of associated bacterial strains. *Spongia officinalis* was collected from Mazara del Vallo (Sicilia, Italy), from a depth of 10 m. The sponge sample was transferred soon after collection to a sterile falcon tube and transported under frozen condition to the laboratory for the isolation of associated microbes. The sponge was then mixed with sterile saline water and vortexed. A small fraction of the liquid was serially diluted up to 10^{-3} dilutions and then spread on plates of Tryptone Yeast agar (TY). The plates were incubated at 37°C for 2 days till growth of colonies was observed. Single bacterial colonies were isolated on the basis of distinct colony morphologies from the TY plates. Isolates were maintained on TY agar plates at 4°C until use.

Supernatant preparation

The isolated bacteria were sub-cultured on M63 (minimal medium) agar plates and incubated at 37°C for two days. A loopful of the bacterial culture from each plate was inoculated into M63 broth (in duplicate), incubated at 37°C for 24 h and then centrifuged at 7000×g for 20 minutes to separate the cell pellets from the fermentation medium. The supernatants were filtered through 0.2µm-pore-size Minisart filters (Sartorius, Hannover, Germany). To ensure that no cells were present in the filtrates, 100 µl were spread onto TY agar plates, and 200 µl were inoculated in separate wells in the microtiter plate.

Screening for bioactive metabolites for biofilm inhibition

Filtered cell free supernatants from the marine sponge-associated isolates were used to test the effect against biofilm formation of *E. coli* PHL628. The same method was used stated above for the biofilm inhibition assay.

Identification and purification of anti-biofilm compound

144 ml of cell free bacterial broth cultures were extensively dialyzed against water for two days, using a membrane tube of 12000-14000 cut-off; this procedure allowed us to remove the large amount of glycerol in the bacterial broth as confirmed by ^{1}H- ^{13}C-NMR experiments recorded on lyophilized broth before and after dialysis; the inner dialysate (25 mg) was fractionated by gel filtration on Sepharose CL6B, eluting with water. Column fractions were analyzed and pooled according to the presence of saccharidic compounds, proteins and nucleic acids. Fractions were tested for carbohydrate qualitatively by spot test on TLC sprayed with α-naphthol and quantitatively by the Dubois method (Dubois *et al.* 1956). Protein content was estimated grossly by spot test on TLC sprayed with ninhydrin and by reading the column fractions absorbance at 280 nm. The active fractions were tested by the Bio-Rad Protein System, with the bovine serum albumin as standard (Bradford, 1976). Finally, the presence of nucleic acids was checked by analysis of fractions absorbance at 260 nm. Furthermore, the grouped fractions were investigated by ^{1}H-NMR spectroscopy. ^{1}H and ^{13}C NMR spectra, were recorded at 600.13MHz on a BrukerDRX-600 spectrometer, equipped with a TCI CryoProbeTM, fitted with a gradient along the Z-axis, whereas for ^{31}P-NMR spectra a Bruker DRX-400 spectrometer was used.

The gel filtration fractions were tested for anti-biofilm activity and the active fraction resulted positive to carbohydrate tests; this latter was a homogenous polysaccharide (6.6 mg) material. Preliminary spectroscopic investigations indicated the presence of a compound with a simple primary structure; the molecular mass of polysaccharidic molecule was estimated by gel filtration on a Sepharose CL6B which had previously been calibrated by dextrans (with a Mw from 10 to 2000 kDa). It's worthy to notice that some resonances in ^{13}C NMR spectrum (78.32, 70.76, 65.63, 67.15 ppm) were split; this suggested the presence of ^{31}P (J_{C-P} from 4 to 9 Hz, table 3.1) and its position into the polysaccharide repeating unit.

The phosphate substitution was confirmed by recording a ^{31}P-NMR spectrum; it showed a single resonance at 1.269 ppm (Rundlöf *et al.* 1996).

The GC-MS analysis of the high-molecular-weight polymer was carried out on an ion-trap MS instrument in EI mode (70eV) (Thermo, Polaris Q) connected with a GC system (Thermo, GCQ) by a 5% diphenyl (30 m x 0.25 mm x 0.25 um) column using helium as gas carrier. Nuclear Overhauser enhancement spectroscopy experiments (NOESY) were acquired using a mixing time of 100 and 150 ms. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 68 ms.

Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain. Experiments were carried

out in the phase-sensitive mode and 50 and 83 ms delays were used for the evolution of long-range connectivities in the HMBC experiment. The 2D ^{1}H–^{31}P HSQC experiment was recorded setting the coupling constants at 10 and 20Hz.

Table 3.1: ^{1}H, ^{13}C and ^{31}P NMR chemical shift of polysaccharide (p.p.m). Spectra in D_2O were measured at 27°C and referenced to internal sodium 3-(trimethylsilyl)-(2,2,3,3-$^{2}H_4$) propionate (δ_H 0.00), internal methanol (δ_C 49.00)and to external aq, 85% (v/v) phosphoric acid (δ_P 0.00)

Residue	**Nuc--leus**	**1**	**2**	**3**	**4**	**5**	**6**
⇾4)-α-D-Galp-(1⇾							
	^{1}H	5.071 H3Gro (3.7 Hz)[a]	3.690	3.784	*3.827* C6,4Gal	*3.917* C3Gal	3.671 H1Gal
	^{13}C	*99.47* H1Gro	69.37	69.95	*78.32* H1Gal (7.8 Hz)[b]	70.19	*62.18* H5Gal
Gro-1-P-(O⇾							
	^{1}H	*3.865* C4Gal -3.906	*4.120* C3,5Gro	3.839-3.770			
	^{13}C	*65.63** H1Gro (4 Hz)[a] - 65.41* (4.5)[a]	*70.76* H1Gal (7.9 Hz)[c]	67.15 (~2 Hz)[d]			
	^{31}P	1.269					

*diastereotopic carbons ; [a] $^{3}J_{H1,H2}$; [b] $^{2}J_{C-P}$; [c] $^{3}J_{C-P}$; [d] $^{4}J_{C-P}$; in italics, the signals showing C–H long-range correlations with the positions in superscripts; Underlined are the NOE contacts with positions in superscripts.

Growth curve analysis

The effect of the bioactive compound on the planktonic culture was checked by growth curve analysis on both *E. coli* PHL628 and *Pseudomonas fluorescens*. The supernatant of the isolate was added to a conical flask containing 50 ml of M63 broth, to which a 1% inoculum from the overnight culture was added. The flask was incubated at 37°C. Growth medium with the addition of bacterial inoculum and without the addition of the supernatant was used as a control. OD values were recorded for up to 24 h at 1-h intervals.

Antibacterial activity by disk diffusion assay

Antimicrobial activity of the supernatant was assayed by the disc diffusion susceptibility test (Clinical and Laboratory Standards Institute, 2006). The disc diffusion test was performed in Muller–Hinton agar (MHA). Overnight cultures of *E. coli* PHL628 and *P. fluorescens* were subcultured in TY broth until a turbidity of 0.5 McFarland (1×10^8 CFU ml^{-1}) was reached. Using a sterile cotton swab, the culture was uniformly spread over the surface of the agar plate. Absorption of excess moisture was allowed to occur for 10 minutes. Then sterile discs with a diameter of 10 mm were placed over the swabbed plates and 50 µl of the extracts were loaded on to the disc. MHA plates were then incubated at 37°C and the zone of inhibition was measured after 24 h.

Microscopic techniques

For visualization of the effect of the sponge-associated bacterial supernatant against the biofilm forming *E. coli* PHL628 and *P. fluorescens*, the biofilms were allowed to grow on glass pieces (1×1 cm) placed in 6-well cell culture plate (*Greiner Bio-one, Frickenhausen, Germany*). The supernatant at concentrations ranging from 1 to 10 times were added in $M63K_{10}$ (for *E. coli* PHL628) and M63 broth (for *P. fluorescens*) containing the bacterial suspension of 0.05 O.D. at 600 nm. The wells without supernatant were used as control.

The plate was incubated for 36 h at 30°C in static condition. After incubation, each well was treated with 0.4% crystal violet for 45 minutes. Stained glass pieces were placed on slides with the bio-film pointing up and were inspected by light microscopy at magnifications of ×40. Visible bio-films were documented with an attached digital camera (*Nikon Eclipse Ti 100*).

Anti-biofilm effect on various strains and growth conditions

Some laboratory strains such as *Acinetobacter*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhimurium*, *Shigella sonneii*, *Listeria monocytogenes*, *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Bacillus pumilus* and *Bacillus subtilis* were selected. All strains were grown in Tryptone Soya Broth (TSB) (Sigma) supplemented with 0.25% glucose and the same medium was used during the biofilm assay in the presence of SP1 supernatant.

Competitiveness between quorum sensing factors and SP1 supernatant

For this experiment the *E. coli* PHL628 supernatant was prepared by using the same conditions as for that of the sponge-isolated strain. Equal volumes of the two supernatants were added either in combination or alone in the microtiter plate containing a culture of *E. coli* PHL628 at an initial turbidity of 0.05 at 600 nm and biofilm formation was measured as described above. Each result was an average of at least 6 replicate wells.

Pre-coating of microtiter plate

Wells were treated with 200 µl of the *B. licheniformis* supernatant for 24 h and then the un-adsorbed supernatant was withdrawn from the wells. Such pre-coated wells were inoculated with *E. coli* PHL628 cultures having an OD of 0.05 at 600 nm. In another set of wells that were not coated with the supernatant, the fresh culture of *E. coli PHL628* having the same density mentioned above were added together with the supernatant (5% v/v). The microtiter plate was then incubated for 36 h in static conditions and biofilm formation was estimated. The control experiments were carried out in wells that were not pre-coated or initially added with the supernatant. Each result was an average of at least 6 replicate wells and three independent experiments.

In a parallel microtiter plate, the supernatant was added to the 36-h biofilm culture in the microtiter plate and was then left at 30°C in static conditions for another 24 h. The experiment was repeated six times to validate the results statistically.

Microbial cell surface hydrophobicity (CSH) assay

Hydrophobicity of the culture of *E. coli* PHL628 and *P. fluorescens* were determined by using MATH (microbial adhesion to hydrocarbons) assay as a measure of their adherence to the hydrophobic hydrocarbon (toluene) following the procedure described by Courtney *et al.* 2009. Briefly, 1ml of bacterial culture (OD530 nm = 1.0) was placed into glass tubes and 100 µl of toluene along with the supernatant (5% v/v) was added. The mixtures were vigorously vortexed for 2 min, incubated 10-min at room temperature to allow phase separation, then the OD530 nm of the lower, aqueous phase was recorded. Controls consisted of cells alone incubated with toluene. The percentage of hydrophobicity was calculated according to the formula: % hydrophobicity = [1–(OD530nm after vortexing/OD530 nm before vortexing)]×100.

Chapter 4:

Results and Discussion

Effect of compounds from Spongia officinalis on E. coli PHL628 *biofilm*

Among 13 purified compounds from the Mediterranean sponge, *Spongia officinalis*, Tetrahydrofurospongin-2 and dihydrofurospongin-2 showed a significant biofilm induction activity. Even in a lower concentration (50 µg/ml) tetrahydrofurospongin-2 and dihydrofurospongin-2 induced the biofilm formation by *E. coli* PHL628 by a factor of 1.57 and 1.93 respectively. However, as the concentration increases, tetrahydrofurospongin-2 becomes more efficient in inducing biofilm. Instead the increase of dihydrofurospongin-2 concentration over 50µg/ml had no additional effect on the biofilm induction.

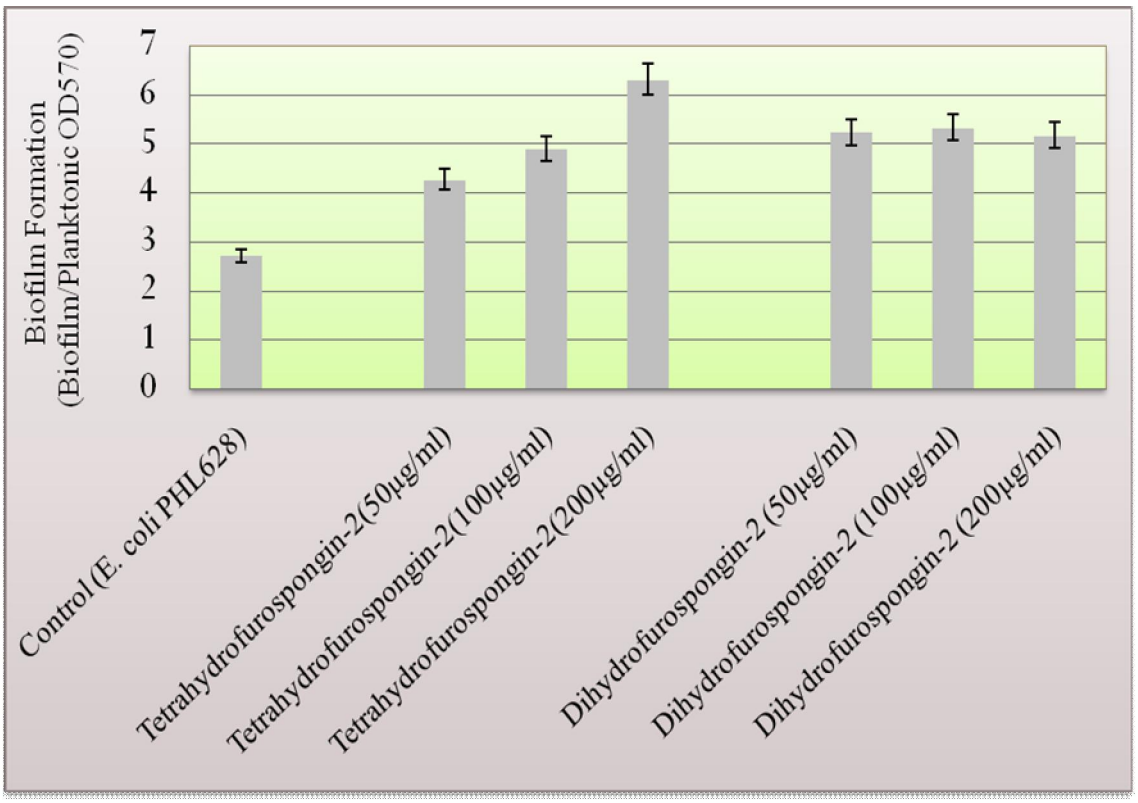


Figure 4.1: Biofilm formed by *Escherichia coli* PHL628 when incubated in presence or absence of tetrahydrofurospongin-2 and dihydrofurospongin-2 in microtiter wells. The plate was incubated for 36 h, followed by crystal violet staining and

spectrophotometric absorbance measurements (OD570). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value is presented as the "biofilm formation" on the *y axis*. Bars represent means ± standard errors for six replicates.

Bacillus licheniformis culture supernatant inhibits biofilm formation by *Escherichia coli* PHL628

Starting from the Mediterranean *Spongia officinalis* sample, it has been possible to distinguish ten different types of prominent colonies in terms of cell shape, size and pigmentation from one hundred colonies of sponge-associated bacteria. They were screened for production of bioactive anti-biofilm metabolites. One colony for each phenotype was grown till stationary phase and the filtered cell-free supernatants obtained were used at a concentration of 3% (v/v) against a stationary culture of the indicator strain *E. coli* PHL628 (Figure 4.2). Supernatants derived from strains SP1 and SP3 showed a strong anti-biofilm activity (65% and 50% reduction, respectively) against *E. coli* PHL628.

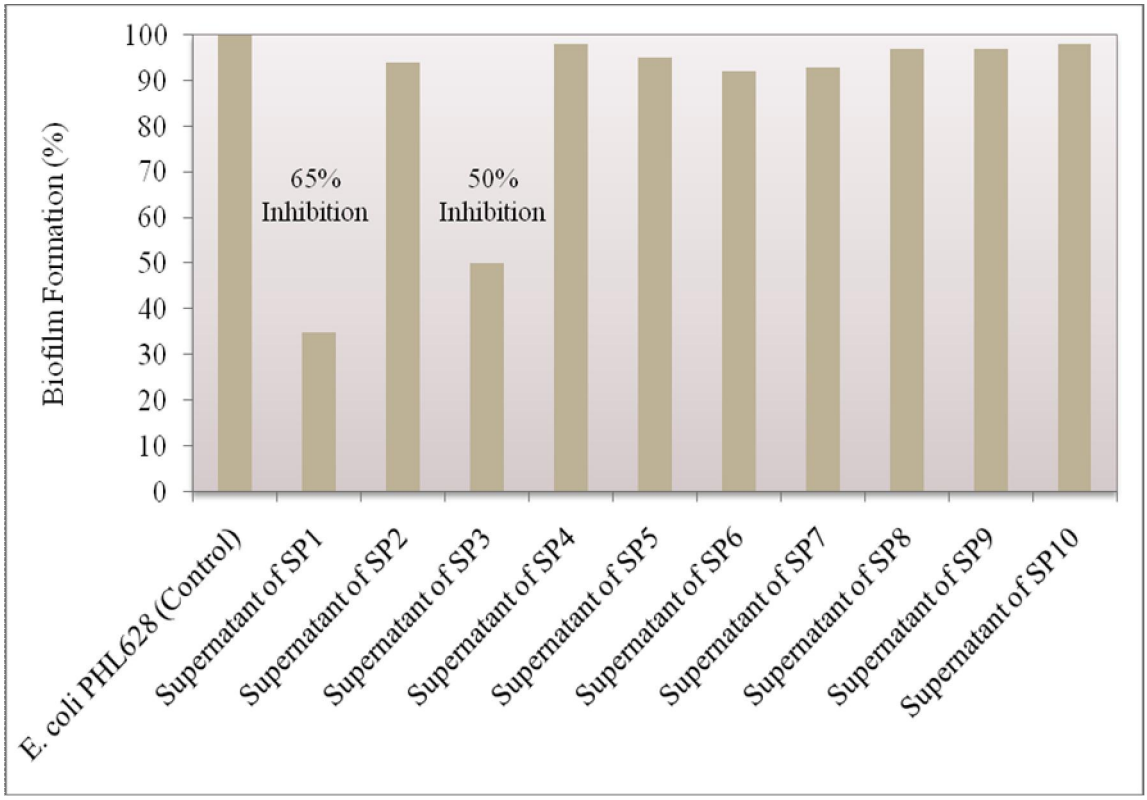


Figure 4.2: Anti-biofilm activity of supernatants from different strains (SP1-SP10) associated with *Spongia officinalis*. Biofilms of *Escherichia coli* PHL628 were allowed to develop in the presence of supernatants (3% v/v) from marine sponge-associated isolates in 96 well microtiter plate. The plate was incubated at 30°C for 36 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD_{570}). The absorbance was used to calculate the "biofilm formation" on the *y axis*. *X axis* represents cell free supernatants from different *Spongia officinalis* isolates. The 100% is represented by *E. coli* PHL628 produced biofilm.

SP1 isolate was chosen to study the nature of the biofilm inhibition mechanism. Sequencing of the 16S RNA revealed that the SP1 gene showed 99% similarity with *Bacillus licheniformis*.

Isolation and purification of active compounds

The active fraction of SP1 cell free supernatant was initially found to be of polysaccharidic composition. Preliminary spectroscopic investigations indicated the presence of a compound with a simple primary structure; the ^{1}H and ^{13}C NMR spectra suggested that the polymer was composed by a regular-repeating unit; the monosaccharide was identified as an acetylated O-methyl glycoside derivative and the compositional analysis was completed by the methylation data which indicated the presence of 4-substituted galactose; in fact the sample was methylated with iodomethane, hydrolized with 2M triofluoroacetic acid (100°C, 2h), the carbonyl was reduced by $NaBD_4$, acetylated with acetic anhydride and pyridine, and analyzed by GC-MS. The molecular mass of the polysaccharidic molecule was estimated to be approximately 1800 kDa by gel filtration on a Sepharose CL6B. In TOCSY, DEPT-HSQC, and HSQC-TOCSY experiments, additional signals of a –CHO– and two $–CH_2O–$ spin system proved the presence of not only a galactose residue but also of a glycerol residue (Gro); the relatively deshielded value for the glycerol methylene carbons at 65.6 and 65.4 ppm was consistent with a phosphate substitution at C1 of glycerol. ^{31}P-NMR spectrum confirms the presence of a phosphodiester group.

The position of the phosphate group between the α-D-galactopyranosyl and the glycerol residue was unambiguously confirmed with 2D ^{1}H ^{31}P-HSQC experiments. In fact, correlations between the ^{31}P resonance and H4 (3.827 ppm) of galactose were observed. This fact established the connectivity of the phosphate group to the respective carbon atoms. It follows that the repeating unit contains the phosphate diester fragment. Galactose was present as pyranose ring, as indicated by ^{1}H- and ^{13}C-NMR chemical shifts and by the

HMBC spectrums that showed some typical intra-residual scalar connectivities between H/C (Table 1). The connection between galactose and glycerol into repeating unit was determined using HMBC and NOE effects. The anomeric site (99.47 and 5.071 ppm) of galactose presented long-range correlations with glycerol C2' (70.76 ppm) and H2' (4.120 ppm), and allowed the localization of galactose binding at C2' of glycerol. NOE contacts of anomeric proton at 5.071 ppm with the signal at 3.839 ppm (Gro H23', table 1) confirmed this hypothesis.

Thus, the polysaccharide is composed of α-D-galactopyranosyl-(1→2)-glycerol-phosphate monomeric units (Figure 4.3).

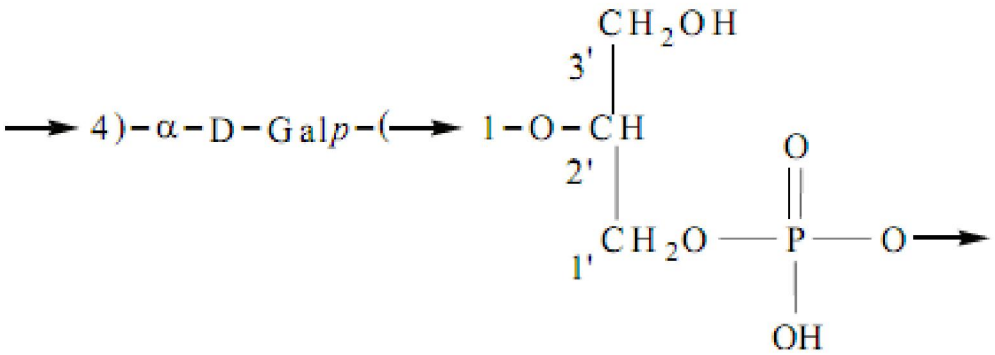
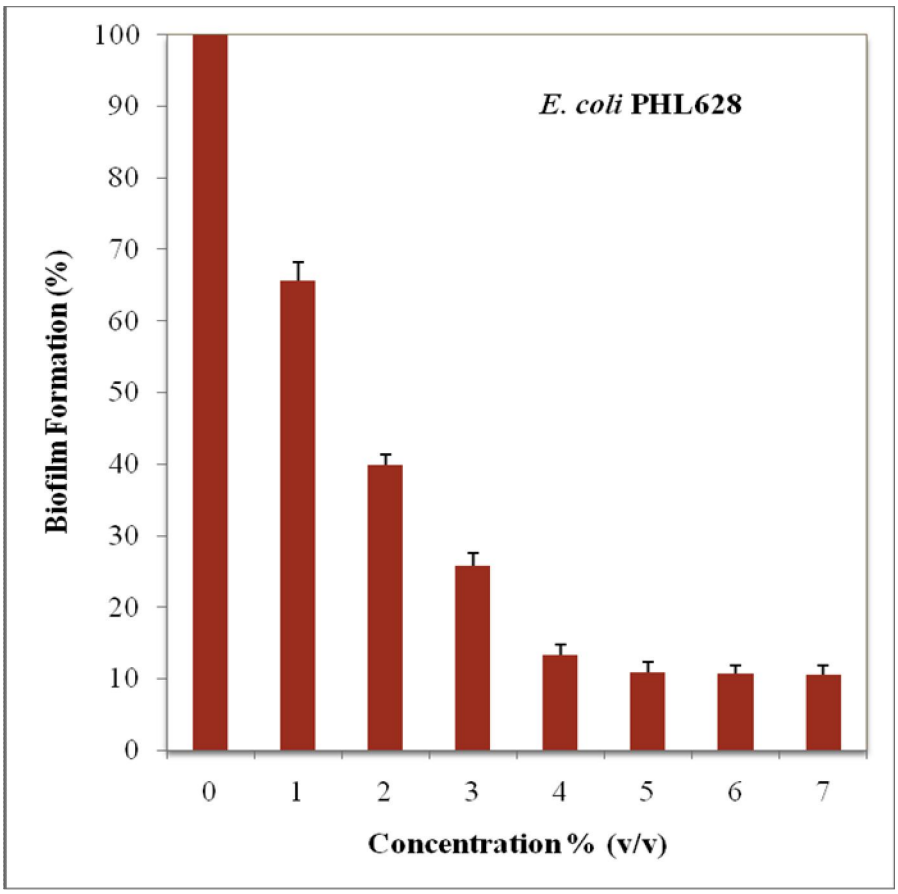


Figure 4.3: Repeating unit of the bacterial polysaccharide having anti-biofilm activity.

Effective concentration for anti-biofilm activity

In order to check whether the anti-biofilm activity of the sponge-associated SP1 strain is dependent on the concentration used in the microtiter plate assay, the cell free supernatant from this strain was tested against biofilm formation by two organisms, *E. coli* PHL628 and *Pseudomonas fluorescens*. The anti-biofilm activity raises as the concentration of the supernatant increases. The anti-biofilm activity of the SP1 supernatant against the two test strains was comparable and perhaps slightly higher for *E. coli* PHL628, as in the presence of 5% (v/v) supernatant, inhibition was about 89% and 80% on biofilm formation by *E. coli* PHL 628 and *Pseudomonas fluorescens*, respectively (Figure 4.4).



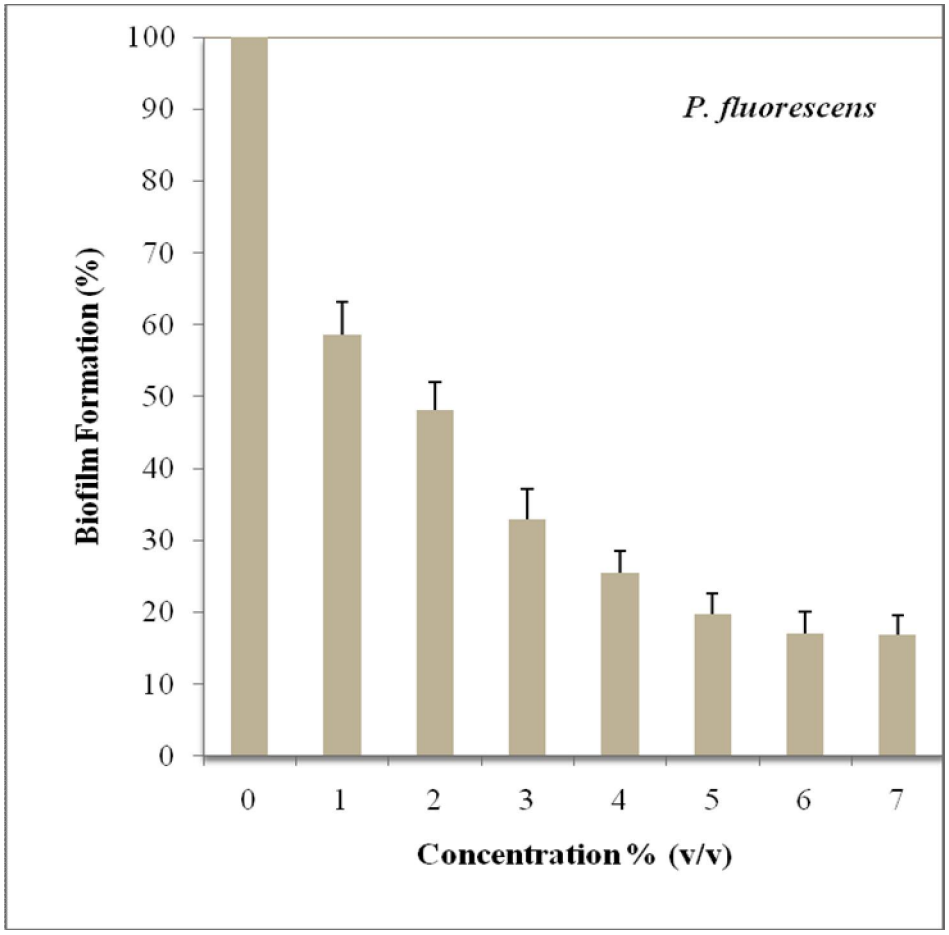


Figure 4.4: Anti-biofilm activity is concentration-dependent. Stationary cells of *E. coli* PHL628 or *P. fluorescens* were incubated along with the SP1 supernatant at different concentrations in 96-well microtiter plate. The plate was incubated at 30°C for 36 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD_{570}). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value was used to calculate the "biofilm formation" on the *y axis*. *X axis* represents the concentration of supernatant used in the wells. Bars represent means ± standard errors for six replicates.

Effect of the supernatant on E. coli and P. fluorescens growth

To evaluate whether the anti-biofilm effect of cell-free supernatant from sponge-associated *B. licheniformis* was related to reduction of growth rate of the target strains, growth curves of both strains were measured in presence and absence of 5% (v/v) supernatant. The resulting growth rates were found to be the same in the two conditions for both *E. coli* PHL628 (0.51±0.02 h^{-1}) and *P. fluorescens* (0.69±0.02 h^{-1}). Growth curves for both strains in presence or absence of supernatants overlapped each other.

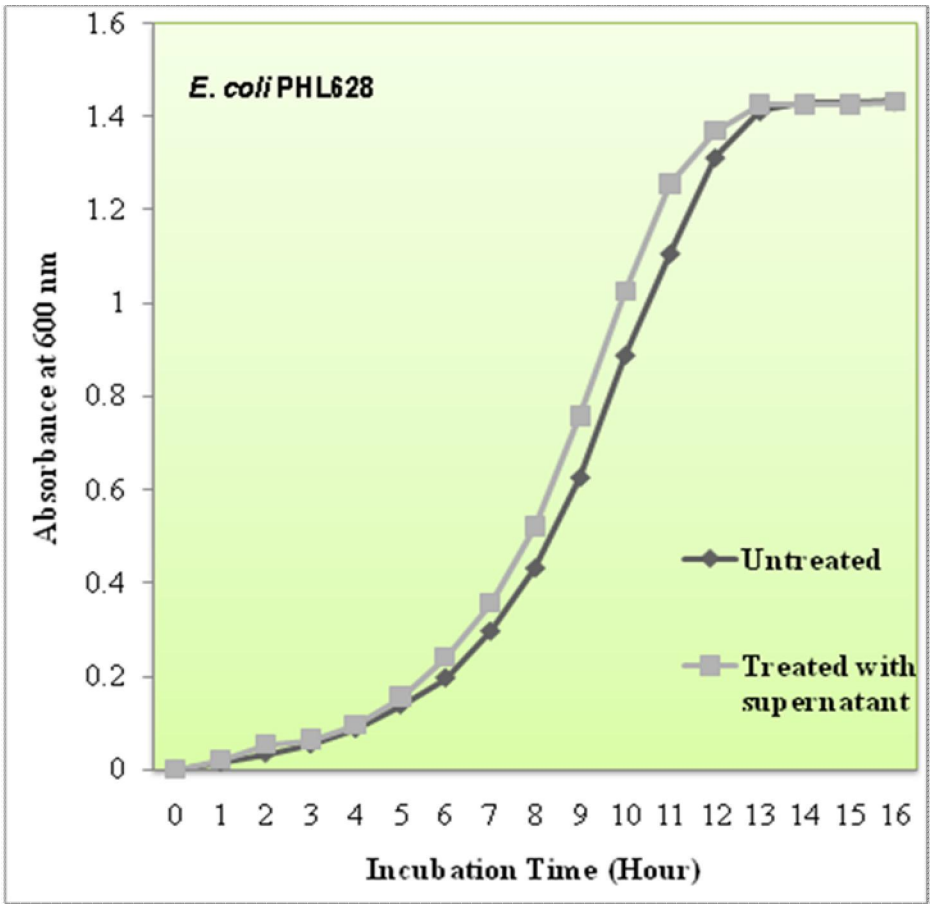


Figure 4.5: Cell free supernatant did not inhibit growth of *E. coli* PHL628. *E. coli* PHL628 were grown in minimal medium, M63K10 in presence or absence of the cell free supernatant at a concentration of 5% (v/v).

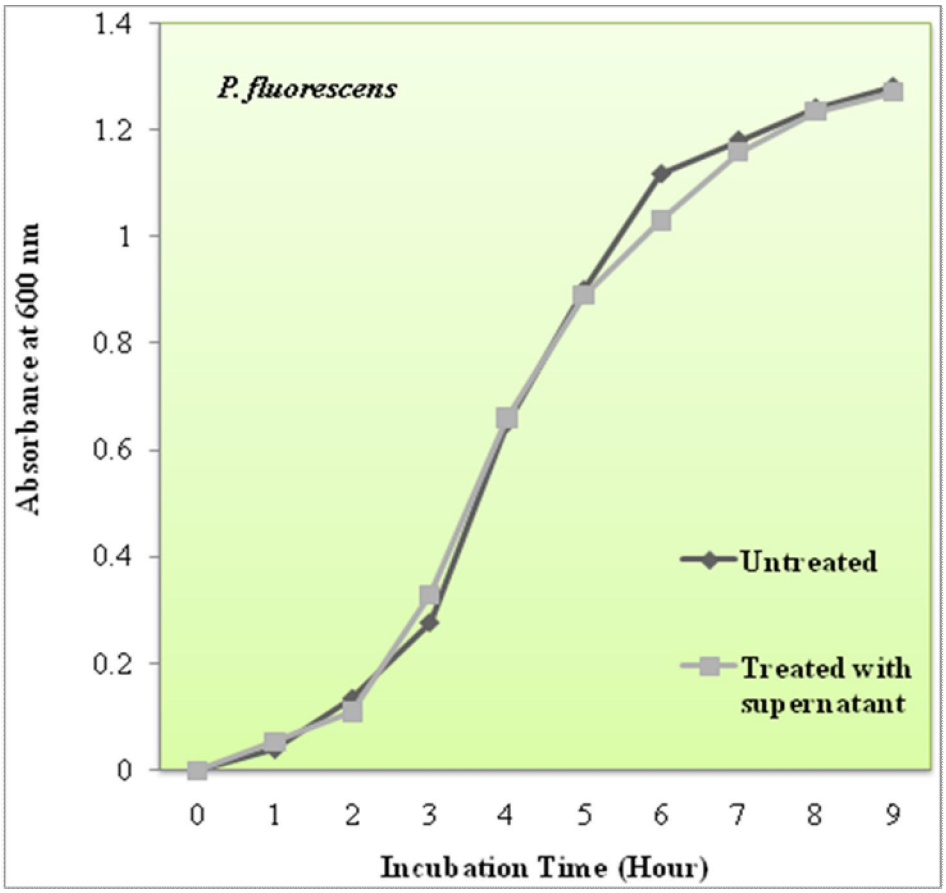


Figure 4.6: Cell free supernatant did not inhibit growth of *P. fluorescens*. *P. fluorescens* were grown in minimal medium, M63 in presence or absence of the cell free supernatant at a concentration of 5% (v/v).

These data were further confirmed by the disc diffusion assay. No inhibition halo surrounding the discs was observed, thereby indicating that the supernatant has no bacteriostatic or bactericidal activity against *E. coli* PHL628 and *P. fluorescens*.

Microscopic visualization of dose dependency for anti-biofilm activity

The efficiency as well as dose dependency of the sponge-associated SP1 supernatant for anti-biofilm activity was evaluated directly by microscopic visualization through the use of coverslips. The use of more concentrated supernatant resulted in less aggregation of cells of both *E. coli* PHL628 and *P. fluorescens* on cover slips as compared to control. Ten-fold concentrated supernatant completely inhibited biofilm formation by *E. coli* PHL628. Very similar effects were observed with *P. fluorescens*.

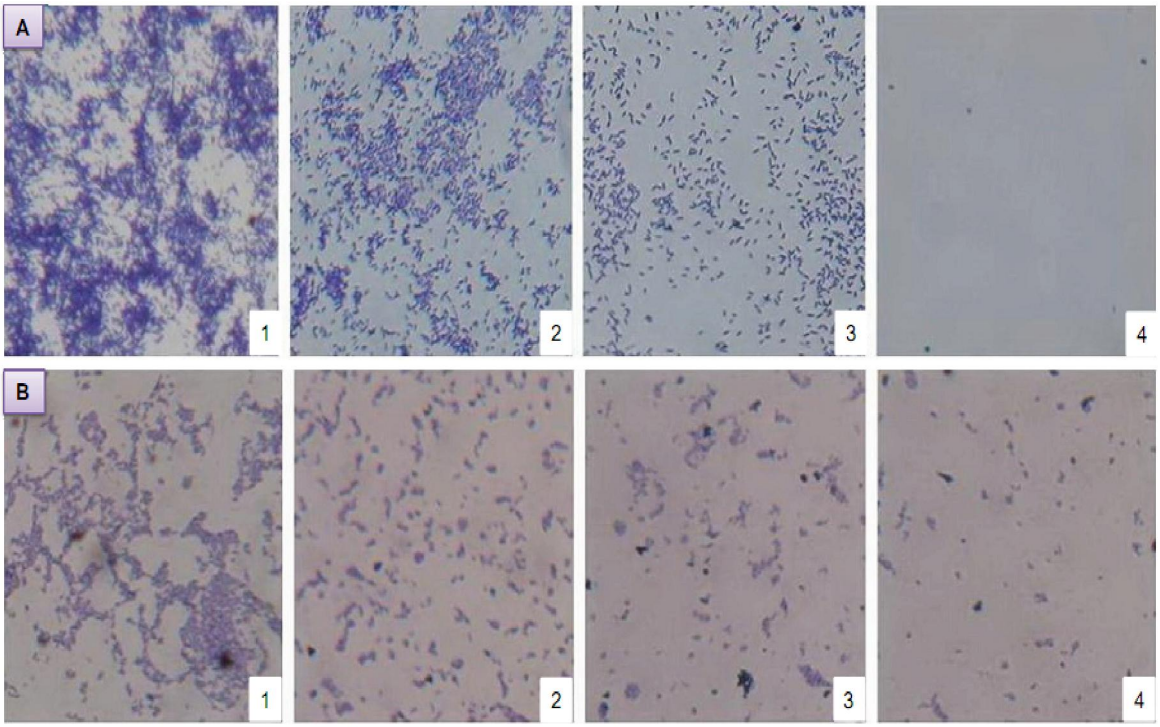


Figure 4.7: Microscope observation of biofilm inhibition. Biofilm inhibition of *E. coli* PHL628 (A) and *Pseudomonas fluorescens* (B) on glass cover slip under a phase-contrast microscope at a magnification of 40X. Bacterial cells were incubated with (1) 1X SP1 supernatant, (2) 2 X SP1 concentrated supernatant, (3) 5 X SP1 concentrated supernatant, (4) 10 X SP1 concentrated supernatant. No difference in biofilm production was observed in the presence of 1X, 2X, 5X and 10X $M63K_{10}$ sterile medium (not shown).

Inhibitory effect of the supernatant on biofilms of various strains

To evaluate further the inhibitory effect of the SP1 supernatant on biofilm development, biofilms of multiple strains comprising both Gram-positive and Gram-negative bacteria were tested with the supernatant. Among the strains, 5 out of 10 appeared to be more than 50% inhibited in their biofilm development by the SP1 supernatant. Very interestingly, in the case of *Staphylococcus aureus*, the inhibition was almost 90%. Among the four *Bacillus* species, *B. amyloliquefaciens* was the most affected one, whereas *B. pumilus* and *B. cereus* were less affected in the inhibition of biofilm development. Not a single strain was stimulated or unaffected in biofilm development by the supernatant.

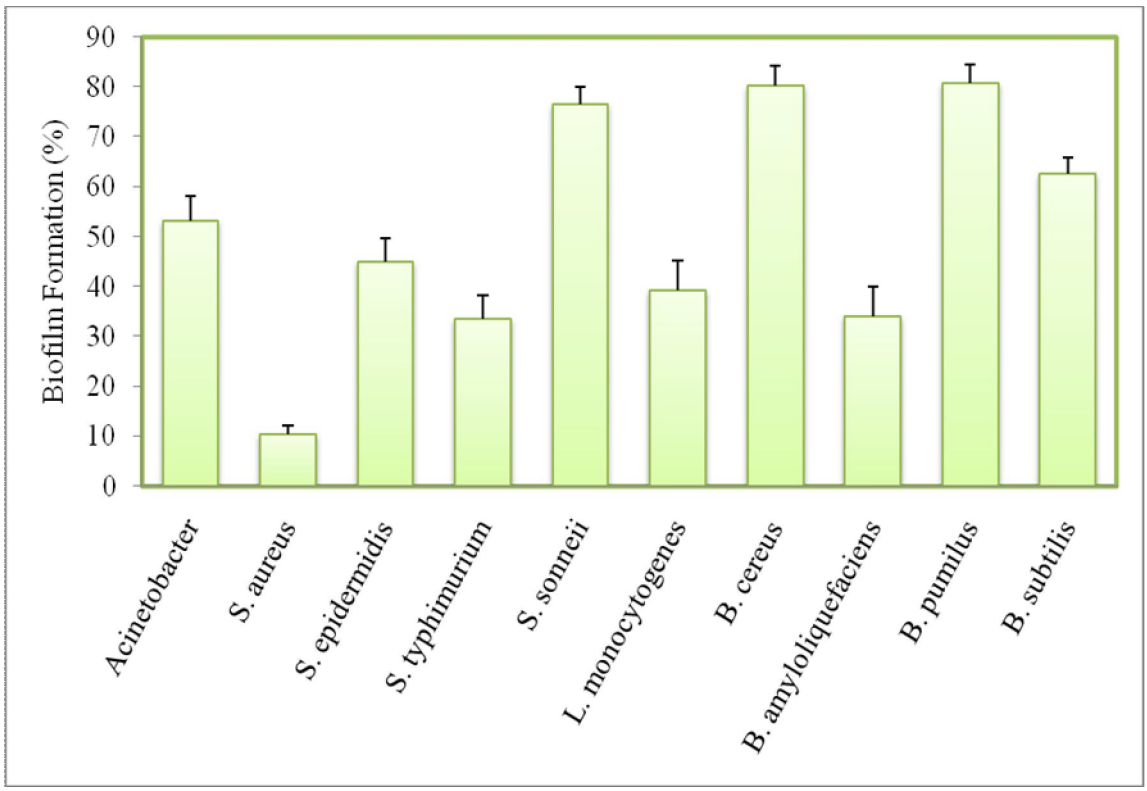


Figure 4.8: Inhibitory effect of the SP1 supernatant over a range of Gram-positive and Gram-negative bacteria. Biofilms of various Gram-positive and Gram-negative

bacteria were developed in the presence or absence of the SP1 supernatant (5% V/v) in 96-well microtiter plate. The plate was incubated at 30°C for 36 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD_{570}). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value used to calculate the "biofilm formation" on the *y axis*. The various Gram-positive and Gram-negative bacteria used in the wells are listed on *X axis*. Bars indicate means ± standard errors for six replicates.

Preliminary characterization of SP1 supernatant

The SP1 cell free supernatant gradually loses its efficiency in decreasing biofilm formation after its pre-treatment at temperatures ranging from 50°C to 80°C. When the supernatant was treated at 50°C, the inhibitory activity towards *E. coli* PHL628 remained 100%, but at 60°C it started to decrease (95%). Treatment at 70°C and 80°C, resulted in 41% and 29% of the anti-biofilm activity respectively. At 90°C the inhibitory activity was completely lost.

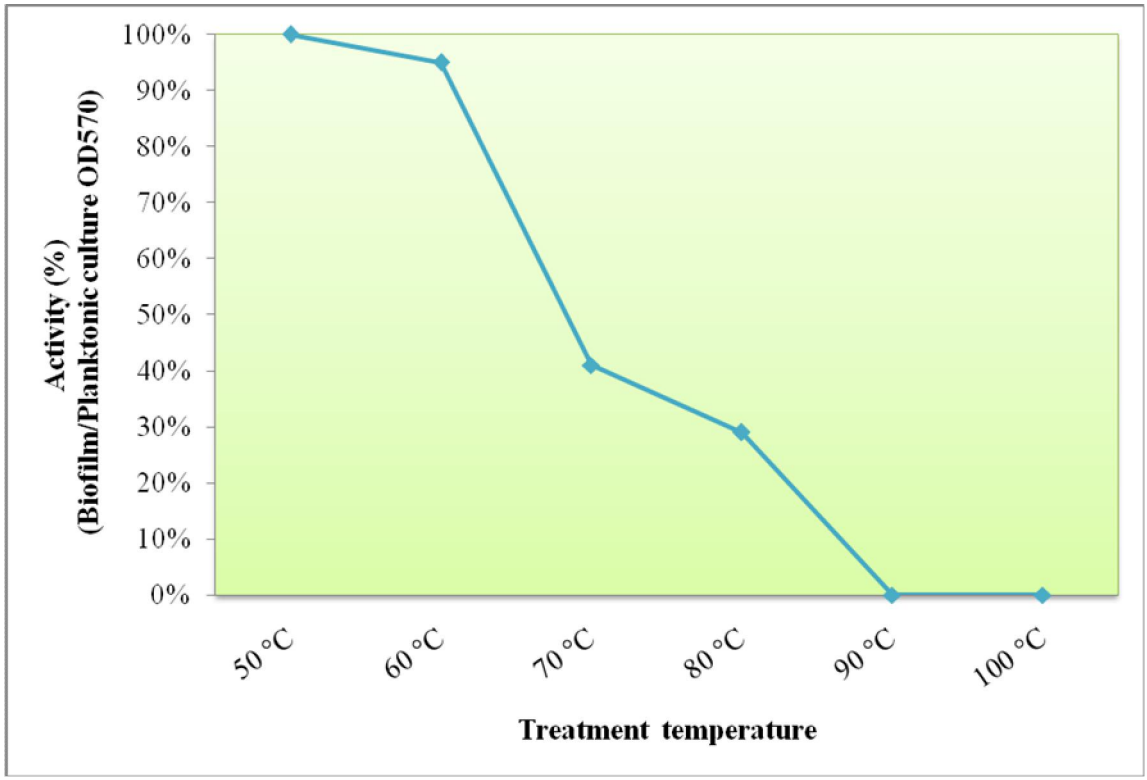


Figure 4.9: Biofilm formed by *E. coli* PHL628 when incubated in presence of supernatant from the isolate treated at different temperatures in microtiter wells. The plate was incubated for 36 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD570). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value is used to calculate the "biofilm inhibition" on the *y axis*. *X axis* represents the treatment temperature of supernatant before use.

Competition of SP1 supernatant with quorum sensing signals for receptor sites

To preliminarily characterize the mechanism of action, SP1 supernatant was compared with the quorum sensing signals obtained from two days supernatant of an *E. coli* PHL628 culture in order to understand if there is a competition for the quorum sensing receptor. The use of the two supernatants together had almost the same effect on biofilm inhibition as the SP1 supernatant alone.

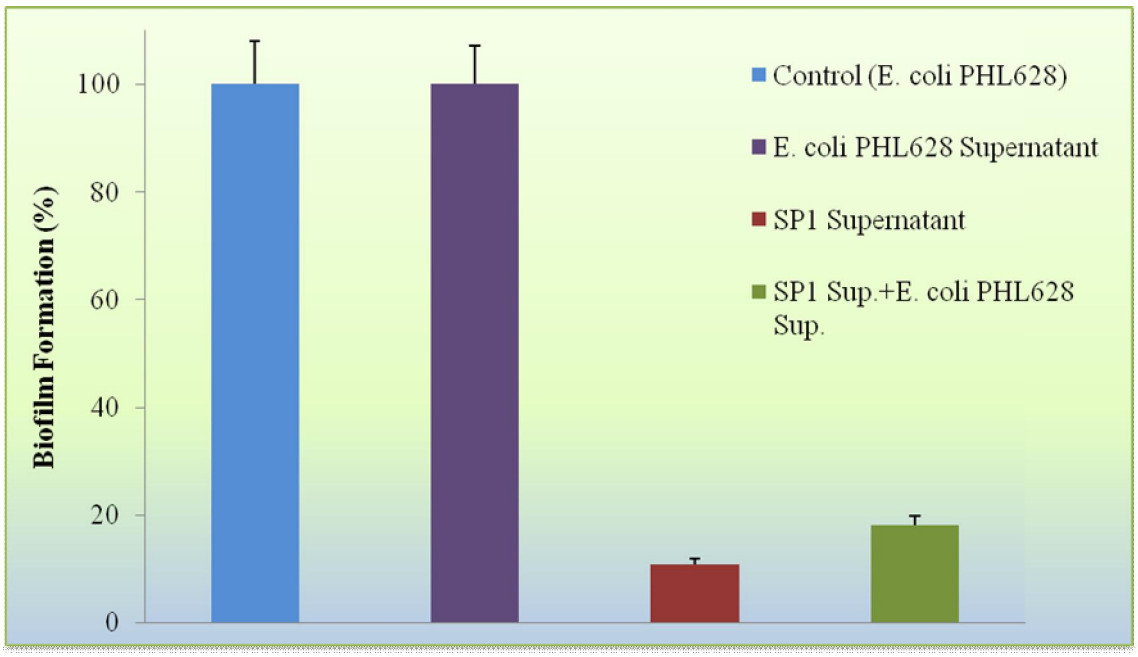


Figure 4.10: Biofilm formed by *E. coli* PHL628 when incubated in presence of various combinations of the supernatants from the isolate and *E. coli* PHL628 in microtiter wells. The plate was incubated for 36 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD570). The ratio of biofilm

absorbance/planktonic absorbance was calculated, and this value is presented as the "biofilm formation" on the *y axis*. Bars represent means ± standard errors for six replicates.

Pre-coating polystyrene wells of microtiter plate with SP1 supernatant

The polysaccharide present in the SP1 supernatant might modify the abiotic surface in such a way that there might be a reduction or inhibition of irreversible attachment of the biofilm forming bacteria to an inanimate object. We tested this hypothesis by analyzing whether there is an effect on biofilm production by *E. coli* PHL628 if the polystyrene wells of the microtiter plate are pre-coated with SP1 supernatant. We observed that after 36 h, while biofilm formation was inhibited by 75% in the un-coated wells and in presence of supernatant, in the pre-coated wells the biofilm assay performed an inhibition of 92.5% (Fig. 7). In addition, to evaluate further the mechanism of action in the initial attachment stage of biofilm development, the supernatant was added in the already formed biofilm. The effects were found to be much lower compared to that of the initial addition or pre-coating of the supernatant in the microtiter wells.

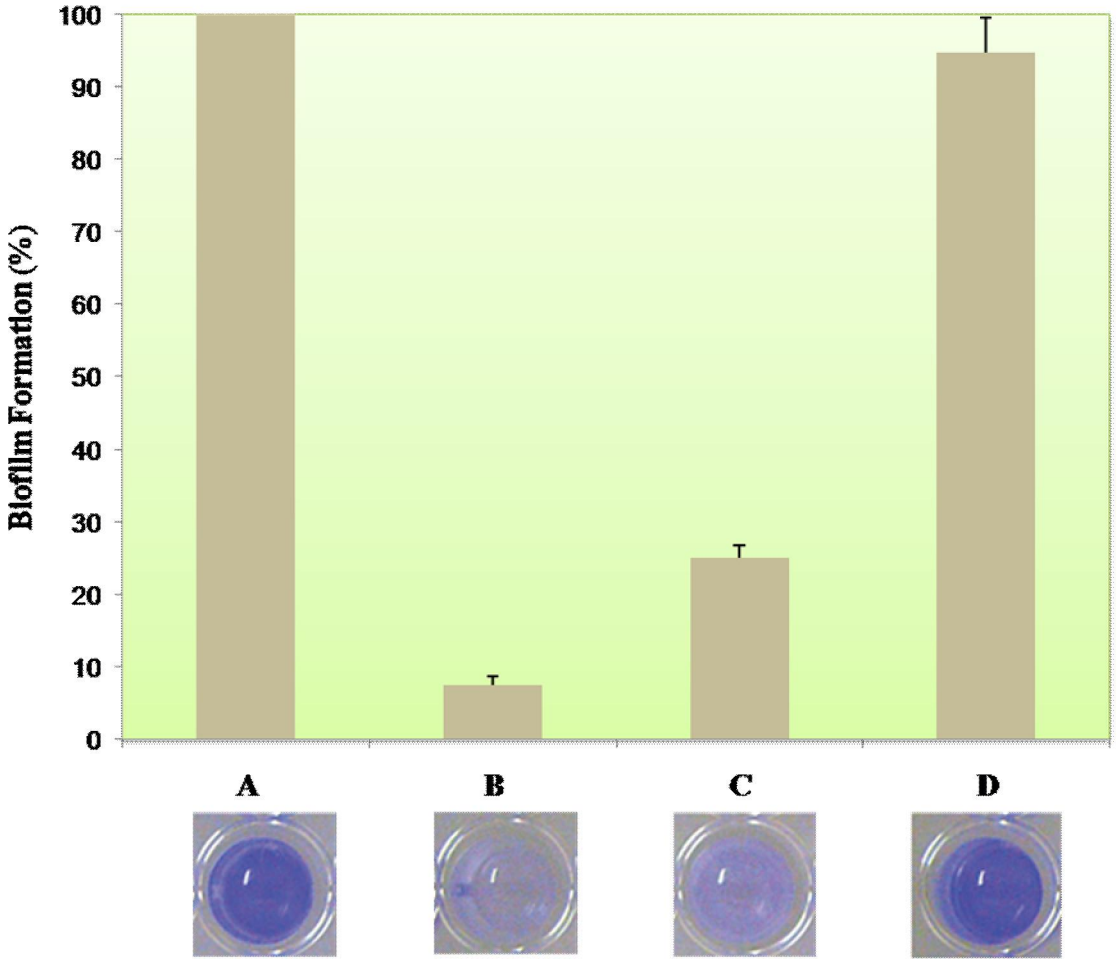


Figure 4.11: Pre-coating with the SP1 supernatant reduces attachment during biofilm formation. Biofilms of *E. coli* PHL628 were developed in 96-well microtiter plates in different conditions: no supernatant (A), wells pre-coated with supernatant (B), supernatant present (C), and supernatant added to pre-formed *E. coli* biofilm (D). The plate was incubated for 36 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD_{570}). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value is presented as the "biofilm formation" on the *y axis*. Bars represent means ± standard errors for six replicates.

Effect on Cell Surface Hydrophobicity (CSH)

Cell surface hydrophobicity (CSH) is important for both adherence and colonization and there is a positive correlation between CSH and biofilm formation (Pompilio *et. al.*, 2008). To analyze whether inhibition of biofilm production is related to reduced adherence of target cells to surfaces, we tested the effects of SP1 supernatant on the degree of cell surface hydrophobicity of *E. coli* PHL628 and *P. fluorescens*. As shown in Figure 4.12, the effects of adding SP1 supernatant to the medium resulted about 60% and 25% decrease in CSH of *E. coli* PHL628 and *P. fluorescens* respectively. Such decrease in CSH might correlate with the reduced adherence and colonization step in biofilm development by both strains in presence of SP1 supernatant.

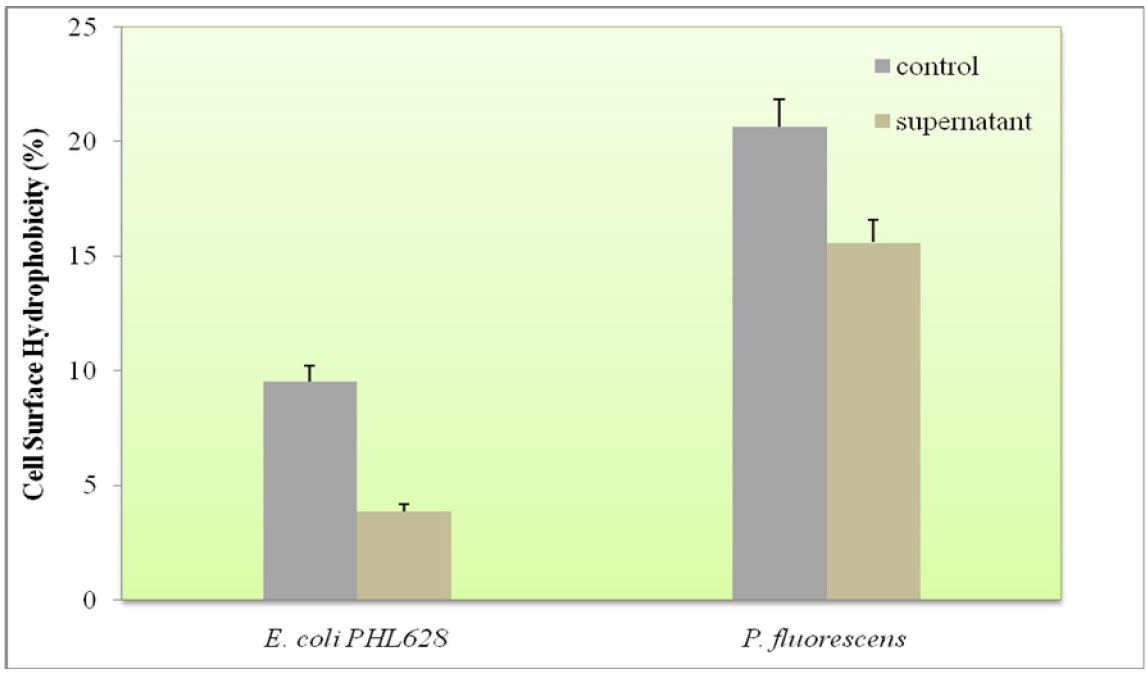


Figure 4.12: Cell surface hydrophobicity (CSH) assay for *E. coli* PHL628 and *P. fluorescens*. *E. coli* PHL628 and *P. fluorescens* were grown in minimal medium $M63K_{10}$ and M63, respectively, in the presence (light tan bars) and absence (gray bars) of SP1 supernatant. Bars represent means ± standard errors for six replicates.

DISCUSSION

Marine biota is a potential source for the isolation of novel anti-biofilm compounds (You *et al.* 2007). Symbiotic associations of bacteria with marine invertebrates play a critical role in the marine environments. It has been estimated that among all the microbes isolated from marine sponge, *Bacillus* species are the most frequently found members so far (Kennedy *et al.* 2009). Therefore the isolation and identification, in the present study, of either the chemical compounds from sponge for biofilm induction or of a sponge-associated *Bacillus licheniformis* having anti-biofilm activity is not surprising.

To our knowledge, the induction of biofilm formation of *E. coli* PHL628, a well established reference strain for laboratory research, by the tetrahydrofurospongin-2 and dihydrofurospongin-2 is the first report on sponge-produced molecules having bacterial biofilm induction properties. This phenomenon could be related to the symbiosis that marine organisms (i.e., algae and sponges) are able to facilitate for some strains of bacteria to develop biofilm on their surface which in turn prevents biofouling stratification.

The occurrence of anti-biofilm activity alone by a previously uncharacterized polymeric polysaccharide having monomeric structure of galactose-glycerol-phosphate is not common. One previous study had shown that the combined action of α-D-galactopyranosyl-glycerol (floridoside) and isethionic acid (floridoside-isethionic acid complex) from red algae had anti-biofilm effect through quorum sensing inhibition (Kim *et al.* 2007). However, to our knowledge, no literature has ever reported the finding of such

compound having monomeric units of galactose-glycerol-phosphate with anti-biofilm activity from marine or other sources.

The polysaccharide is secreted in the culture supernatant by the sponge-associated *B. licheniformis* and its addition to a range of Gram-positive and Gram-negative bacteria results in negative effect on their biofilm development. This broad spectrum of anti-biofilm activity might help *B. licheniformis* during a competitive edge in the marine environment to establish itself on the surface of host sponges and critically influence the development of unique bacterial community.

It has previously been reported that bacterial extracellular polysaccharides can be involved both in biofilm and anti-biofilm activities. For example EPSs from *V. cholera* containing the neutral sugars glucose and galactose are important architectural components of its biofilm (Yildiz and Schoolnik, 1999; Kierek and Watnick, 2003; Fong *et al.* 2010). On the other hand, EPSs from *E. coli* (group II capsular polysaccharide) (Valle *et al.* 2006), *V. vulnificus* (capsular polysaccharide) (Reddy et al. 1993), *P. aeruginosa* (mainly extracellular polysaccharide) (Qin *et el.* 2009; Pihl *et al.* 2010) and marine bacterium *Vibrio* sp.QY101 (exopolysaccharide) (Jiang *et al.* 2011) display selective or broad spectrum anti-biofilm activity. However, the potentiality of the polysaccharide described in this study over a wide range of pathogenic and non pathogenic organisms suggests that the compound might be a powerful alternative among the previously identified polysaccharides in multispecies biofilm context.

Based on the findings, we hypothesize that the secreted sponge-associated bacterial polysaccharide might interfere with the cell-surface and cell-cell interactions in the initial reversible and irreversible attachment stage, which is the pre-requisite for biofilm development (O'Toole *et al.* 2000).

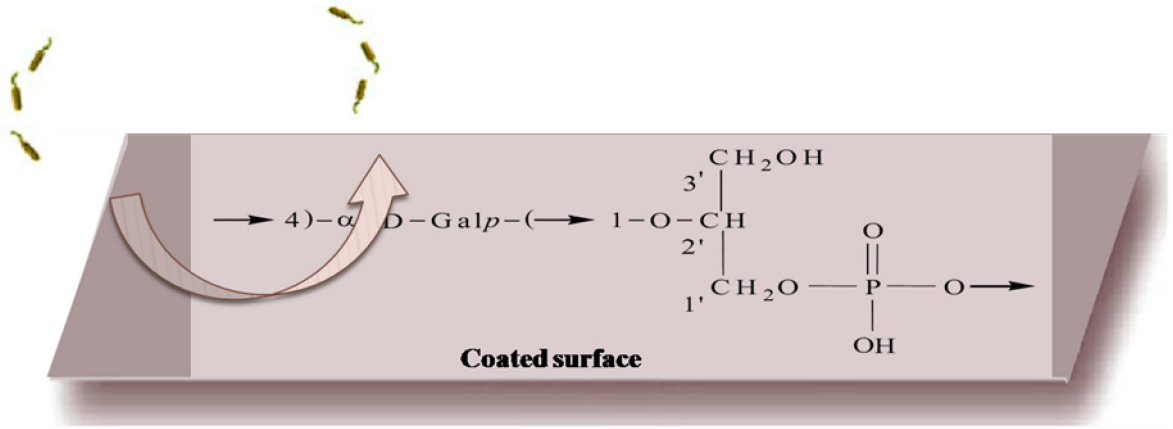


Figure 4.13: Possible inhibition of initial attachment on abiotic surface coated with the secreted compound from sponge-associated *B. licheniformis*.

In a previous study, it was reported that polysaccharides can produce anti-adherence effects between microorganisms and surfaces (Langille *et al.* 2000). The *E. coli* group II CPS and exo-polysaccharides of marine *Vibrio* sp. were reported to inhibit biofilm formation not only by weakening cell-surface contacts but also by reducing cell–cell interactions or disrupting the interactions of cell-surfaces and cell-cell (Valle *et al.* 2006; Jiang *et al.* 2011). In all the previously described polysaccharides having anti-adherence property, highly anionic nature was proposed to be the cause of interference with the adherence properties of cell-surface and cell-cell interaction (Valle *et al.* 2006; Jiang *et al.* 2011; Jann *et al.* 1980). The *B. licheniformis* secreted compound reported here has also high content of phosphate groups and thus it can be proposed that the electronegative

property of the compound might modulate the surface of the tested organism in such a way that there is a reduction or complete inhibition of cell-surface or cell-cell attachment.

It might be possible that the SP1 compound can modify the physicochemical characteristics and the architecture of the outermost surface of biofilm forming organisms which is the phenomenon observed for some antibiotics (Fonseca *et al.* 2004). Reduction of cell surface hydrophobicity of *E. coli* PHL628 and *P. fluorescens* clearly indicates the modification of the cell surface, resulting in reduced colonization and thereby significant contribution to anti-biofilm effect. Almost similar results were obtained with coral-associated bacterial extracts for the anti-biofilm activity against *Streptococcus pyogenes* (Thenmozhi *et al.* 2009).

Anti-biofilm effects were reported to be accompanied in most cases by a loss of cell viability or the presence of quorum sensing analogues. Interestingly, the polysaccharide in the present study is devoid of antibacterial effect, which was demonstrated by the growth curve analysis and disc diffusion test with *E. coli* PHL628 and *P. fluorescens*. An almost similar observation has been reported with the exo-polysaccharide from the marine bacterium *Vibrio* sp. which displayed anti-biofilm nature without decreasing bacterial viability (Jiang *et al.* 2011). However, further experiments suggest that the present polysaccharide enhances the planktonic growth of *E. coli* PHL628 in the microtiter plate wells during biofilm production. Another interesting phenomenon of the bioactive compound reported here is the absence of competition with the quorum sensing signals presumably present in supernatants of the target biofilm-forming bacteria used in this

study. In addition, none of the previously reported quorum sensing competitors is structurally related to the polysaccharide reported here.

In the microscopic visualization experiment through the use of cover slips, biofilm inhibition was also evidenced and was found to display a gradual decrease of biofilm development with the increase of the concentration of the polysaccharide in the culture of *E. coli PHL*628 and *P. fluorescens*. In addition, pre-coating the wells of the polystyrene microtiter plate with the compound also effectively inhibits biofilm formation. There are also some reports on the use of pre-coating surfaces with different surfactants and enzymes (Mireles *et al.* 2001; Kaplan *et al.* 2004; Meriem *et al.* 2011) for anti-biofilm activity.

The present study, however, not only demonstrates the anti-biofilm activity through pre-coating the surface with the polysaccharide from sponge-associated bacteria but also clearly visualize the anti-biofilm nature of the compound through the use of cover slip.

Chapter 5:

Conclusion and Future Prospective

The Mediterranean sponge, *Spongia officinalis*, secretes unique metabolites such as tetrahydrofurospongin-2 and dihydrofurospongin-2 that induce the establishment of biofilms of desired microbial community on their surface. It will be of great benefit to human being if these sponge associated microbial communities are fully explored to find out more novel potential anti-biofilm compounds.

In the present research, the polysaccharide isolated from *Spongia officinalis* associated *B. licheniformis* has several features that provide a tool for better exploration of novel anti-biofilm compounds. First of all, the compound is active against biofilm formation of a wide range of Gram-positive and Gram-negative bacteria. Secondly, the compound has no negative effect on the planktonic growth of already established biofilm forming bacteria.

The present anti-biofilm compound comprising α-D-galactopyranosyl-(1→2)-glycerol-phosphate monomeric units modifies the target surface in such a way that blocks the intial attachment stage which is believed to be crucial in biofilm development. We may also conclude that the physicochemical properties of the cell surface are also altered which in turn may interfere the cell-cell or cell-surface interactions. More intensive researches on this phenomenon are yet to make to provide a better tool in understanding precisely the mechanism of action of the SP1 anti-biofilm compound.

Inhibiting biofilm formation of a wide range of bacteria without affecting their growth represents a special feature of the polysaccharide described in this report. Further research on such surface-active anti-biofilm compounds from sponge-associated marine bacteria might help developing new classes of anti-biofilm molecules with broad spectrum activity and more in general will allow exploring new functions of bacterial polysaccharides in the environment.

Chapter 6:

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Chapter 7:

Appendix

JOURNAL OF NATURAL PRODUCTS

ARTICLE

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Bioactive Terpenes from *Spongia officinalis*

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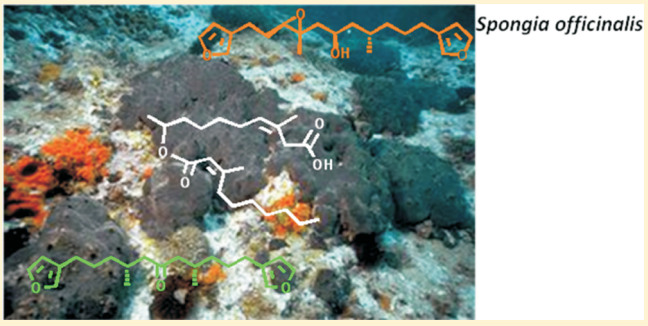
Supporting Information

ABSTRACT: The terpene metabolite pattern of Mediterranean *Spongia officinalis* was chemically investigated. This study resulted in the isolation of a series of sesterterpenes and C_{21} furanoterpenes, according to the literature data on this sponge. Four new oxidized minor metabolites (compounds **1**, **2**, **3**, and **4**) were isolated along with six known compounds of the furospongin series (compounds **5**−**8**, **9**, and **10**) and three scalarane sesterterpenes (compounds **11**−**13**). Interestingly, tetrahydrofurospongin-2 (**6**) and dihydrofurospongin-2 (**7**), which were among the main metabolites, induced biofilm formation by *Escherichia coli*. All compounds isolated were also assayed for antibacterial and antifungal properties.



Marine sponges have been the focus of much recent interest due to two main reasons: (a) they are a rich source of bioactive secondary metabolites[1] and (b) they form close associations with a wide variety of microorganisms.[2] These two facts are strictly related because in several cases the production of the bioactive molecules isolated from the sponges can be ascribed to the associated bacteria.[3] This increasing research interest has greatly improved our knowledge about the communication between sponges and their microbial associates even though many gaps remain in the understanding of such interactions. An interesting aspect of studies in this field is the chemistry of bacterial biofilms and in particular the identification of the molecules that could mediate by either induction or inhibition such sponge−microbe interactions.[4] In this light, in the continuation of our studies on bioactive compounds from marine organisms, we have investigated the chemistry of a specimen of Mediterranean *Spongia officinalis* (Spongiidae), collected off the Sicily coast (Mazara del Vallo). Some selected terpene fractions from the ether extract of the sponge were observed to induce biofilm formation by *Escherichia coli*.

Previous chemical studies on *S. officinalis* from different sites have resulted in the isolation of linear furanosesterterpenes, C_{21} furanoterpenes, and scalarane sesterterpenes.[5] These groups of compounds have also been reported from other genera of Spongiidae families[5a,c,6] and from nudibranch mollusks feeding on them.[7]

The terpene metabolites identified in this study included all three groups of compounds. Four new molecules were chemically characterized including a C_{21} furanoterpene, 7,8-epoxyfurospongin-1 (**1**), two linear carboxylic acids [officinoic acid A (**2**) and officinoic acid B (**3**)], and a linear furanosesterterpene, isofurospongin-4 (**4**). These molecules were isolated together with five known C_{21} furanoterpenes of the furospongin series, compounds **5**−**9**, the linear furanosesterterpene **10**, and three scalarane sesterterpenes, **11**−**13**. Tetrahydrofurospongin-2 (**6**) and dihydrofurospongin-2 (**7**), which were among the main metabolites of the extract, were shown to be responsible for the biofilm induction activity observed in two selected crude terpene fractions of the extract.

RESULTS AND DISCUSSION

The Et_2O-soluble portion from the acetone extract of *S. officinalis* was fractionated by a silica gel column using a light petroleum ether/Et_2O gradient system with increasing polarity as eluent. By this chromatographic step, four fewer polar furospongin metabolites, anhydrofurospongin-1 (**5**),[5c] tetrahydrofurospongin-2 (**6**),[5c] dihydrofurospongin-2 (**7**),[5c] and furospongin-1 (**8**),[5a] were obtained with only minor impurities. Compounds **6** and **7** were further purified by reversed-phase HPLC chromatography. An additional, more polar fraction constituted of a complex mixture of terpenes was also recovered from the silica gel column and subsequently submitted to further purification steps by silica gel and reversed-phase HPLC chromatography to give 7,8-epoxyfurospongin-1 (**1**), furospongolide (**9**),[8] furospongin-4 (**10**),[5d] officinoic acid A (**2**), officinoic acid B (**3**), isofurospongin-4 (**4**), 12α-deoxoscalarin (**11**),[5f] 16-deacetoxy-12-epi-scalarafuranacetate

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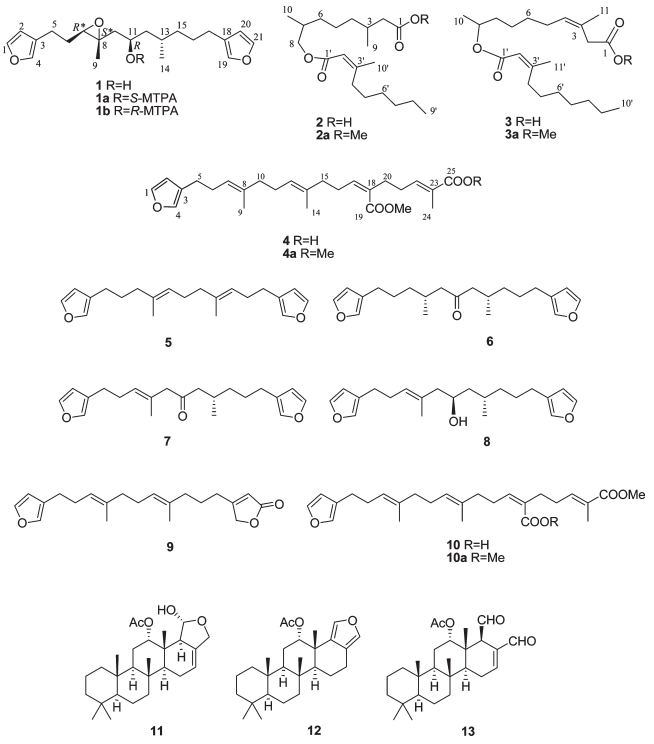
(**12**),[5g] and scalaradial (**13**)[9] (see Experimental Section). Known compounds were identified by comparison of their spectroscopic data with literature values, whereas the structures of unprecedented compounds **1**, **2**, **3**, and **4** were established as follows.



A preliminary analysis of the NMR spectra of new molecules **1**, **2**, **3**, and **4** showed structural relationships of both **1** and **4** with the co-occurring furanoterpenes **5**−**8**, **9**, and **10**, whereas officinoic acid A (**2**) and officinoic acid B (**3**) appeared to be unrelated to either furanoterpenes or scalarane metabolites.

Compound **1** was isolated as an optically active colorless oil and showed a positive Ehrlich reaction, suggesting a furan ring. It had the molecular formula $C_{21}H_{30}O_4$, as suggested by the sodiated molecular peak at m/z 369.2040 in the HRESIMS spectrum. The ^{13}C and ^{1}H NMR spectra of **1** were consistent with a typical structure of a C_{21} difuranoterpene (Table 1) and showed close similarities with those of co-occurring furospongin-1 (**8**),[5a,10] suggesting a related structure. The only difference was the presence of an epoxide ring in **1** replacing the double bond at C-7 in **8**. In fact, the ^{1}H NMR spectrum of **1** contained a methyl singlet at δ 1.30 (H_3-9) and an epoxide proton signal at δ 2.80 (t, J = 6.2 Hz, H-7) rather than the vinyl signals due to the trisubstituted double bond present in **8**. Accordingly, in the ^{13}C NMR spectrum of **1** two oxygenated carbon signals at δ 63.2 (CH, C-7) and 60.9 (C, C-8) (Table 1) were observed in the place of the olefinic carbons in **8**.[10] Thus compound **1** was 7,8-epoxyfurospongin-1. The structure was confirmed by 2D-NMR data. The relative configuration of the epoxide moiety was defined as drawn in formula **1** by NOE difference experiments. Steric effects were observed between the methylene at δ 1.81 (H_2-6) and the singlet methyl at δ 1.30 (H_3-9) as well as between the epoxide proton at δ 2.80 (H-7) and the methylene at δ 1.75 (H-10a) and 1.46 (H-10b). The absolute configuration at C-11 was established by applying the modified Mosher's method. (*S*)- and (*R*)-MTPA esters of **1** were obtained by treating **1** with

Table 1. NMR Spectroscopic Data[a] for 7,8-Epoxyfurospongin-1 (1)

position	δ_C, type	δ_H (J in Hz)	HMBC[b]
1	142.7, CH	7.37, bs	2, 4
2	110.7, CH	6.30, bs	1
3	124.1, C		2, 4
4	138.9, CH	7.25, bs	1
5a	21.5, CH_2	2.64, m	2, 6
5b		2.59, m	
6	28.8, CH_2	1.81, q (6.2)	5, 7
7	63.2, CH	2.80, t (6.2)	9
8	60.9, C		9
9	17.2, CH_3	1.30, s	7, 10
10a	45.2, CH_2	1.75, m	9, 11
10b		1.46, m	
11	66.7, CH	4.06, m	10, 12
12a	45.2, CH_2	1.48, m	11, 14
12b		1.08, m	
13	28.6, CH	1.71, m	14, 15
14	18.9, CH_3	0.93, d (6.6)	13, 15
15a	37.3, CH_2	1.32, m	13, 14
15b		1.26, m	
16a	26.9, CH_2	1.59, m	15, 17
16b		1.51, m	
17a	24.9, CH_2	2.40, t (7.3)	16, 20
18	125.1, C		17, 19, 20
19	138.5, CH	7.21, bs	21
20	110.7, CH	6.28, bs	19, 21
21	142.3, CH	7.34, bs	19, 20

[a] 600 MHz for ^{1}H NMR spectra and 75.47 MHz for ^{13}C NMR spectra ($CDCl_3$); assignments aided by COSY, HSQC, and HMBC. [b] HMBC correlations, optimized for 10 Hz, are from proton(s) stated to the indicated carbon.

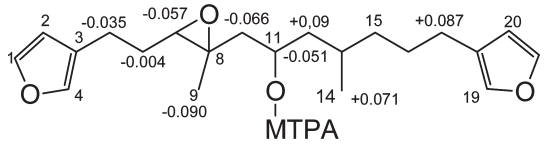


Figure 1. Chemical shift differences ($\Delta\delta = \delta_{S\,\mathrm{ester}} - \delta_{R\,\mathrm{ester}}$) for (*S*)- and (*R*)-MTPA derivatives of compound **1**.

(*R*)- and (*S*)- MTPA chlorides, respectively, and characterized by 2D-NMR experiments [$\Delta\delta$ ($\delta_{S\ \mathrm{ester}} - \delta_{R\ \mathrm{ester}}$) are reported in Figure 1]. The $\Delta\delta$ values observed for the signals of protons close to the hydroxy group at C-11 indicated the *R* configuration, the same as that reported for furospongin-1 (**8**), the absolute configuration of which has been determined by chemical and spectroscopic methods.[5c,6a,11] Accordingly, by biogenetic considerations, the absolute configuration at C-13 in **1** was tentatively assigned to be the same as in **8**.

The molecular formula $C_{20}H_{36}O_4$ of officinoic acid A (**2**) was deduced by the sodiated molecular ion peak at m/z 363.2498 in the HRESIMS spectrum. The IR spectrum showed two intense bands at 1710 and 1651 cm^{-1}, suggesting the presence of two carbonyl groups. This was confirmed by the ^{13}C NMR spectrum, containing signals at δ 174.7 and 167.1 attributable to a

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Table 2. NMR Spectroscopic Data[a] for Officinoic Acids A (2) and B (3)

	2			3		
position	δ_C mult.	δ_H (J in Hz)	HMBC[c]	δ_C mult.	δ_H (J in Hz)	HMBC[c]
1	174.7, C		2	173.8, C		2
2	40.8, CH_2	2.34, dd (15.0, 5.5)	3, 9	36.8, CH_2	3.06, br s	4, 11
		2.16, dd (15.0, 7.6)				
3	30.1, CH	1.95, m	2, 9	127.9, C		2, 4, 11
4	29.6, CH_2	1.30, m	2, 5, 9	129.4, CH	5.36, br t (7.3)	5, 11
5	31.5, CH_2	1.40, m	6	28.2, CH_2	2.01, m	4, 6
6	30.3, CH_2	1.21, m	5, 10	29.3,[b] CH_2	1.32, m	5
7	32.8, CH	1.76, m	6, 10	29.8,[b] CH_2	1.30, m	5
8	68.4, CH_2	3.96, dd (10.6, 6.2)	6, 10	36.0, CH_2	1.60, m	10
		3.87, dd (10.6, 6.6)			1.48, m	
9	19.3, CH_3	0.97, d (6.7)	2, 3	69.9, CH	4.92, dq (6.2,5.5)	8,10
10	17.1, CH_3	0.93, d (6.7)	7	20.1, CH_3	1.21, d (6.2)	8
11				24.0, CH_3	1.80, br s	2,4
1′	167.1, C		10, 2′	165.7, C		9, 2′
2′	115.8, CH	5.65, br s	10′	116.5, CH	5.62, br s	4′, 11′
3′	160.8, C		2′,4′,10′	160.3, C		2′, 4′, 11
4′	33.3, CH_2	2.61, t (7.3)	5′, 10′	33.5, CH_2	2.61, t (7.0)	5′, 11′
5′	28.8, CH_2	1.45, m	4′, 6′	28.0, CH_2	1.48, m	4′
6′	29.6, CH_2	1.32, m	4′	29.2,[b] CH_2	1.30, m	4′
7′	31.9, CH_2	1.31, m	9′	29.3,[b] CH_2	1.30, m	5′
8′	22.4, CH_2	1.28, m	9′	31.8, CH_2	1.30, m	10′
9′	14.2, CH_3	0.88, t (6.2)	8′	22.7, CH_2	1.27, m	10′
10′	25.2, CH_3	1.88, br s	2′, 4′	14.1, CH_3	0.89, t (6.6)	8′, 9′
11′				25.1, CH_3	1.87, s	2′, 4′

[a] 600 MHz for ^{1}H NMR spectra and 75.47 MHz for ^{13}C NMR spectra ($CDCl_3$); assignments aided by COSY, HSQC, and HMBC. [b] Interchangeable values. [c] HMBC correlations, optimized for 10 Hz, are from proton(s) stated to the indicated carbon.

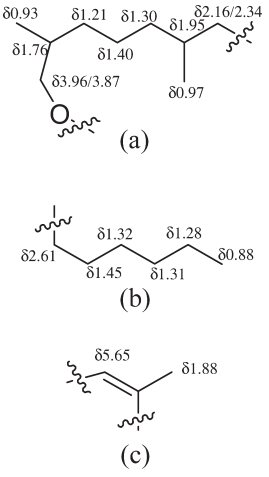

Figure 2. Spin systems for compound **2**.

carboxylic acid and an α,β-unsaturated ester, respectively (Table 2). The carbon spectrum also displayed signals due to a trisubstituted double bond [δ 115.8 (CH, C-2′) and δ 160.8 (C, C-3′)] and 16 sp^3 resonances (4 × CH_3, 10 × CH_2, 2 × CH) including a signal due to an oxygenated methylene [δ 68.4 (CH_2, C-8)], which were consistent with an acyclic carbon skeleton (Table 2). The ^{1}H NMR spectrum showed a vinyl signal at δ 5.65 (1H, br s, H-2′); multiplets at δ 3.96 (1H, dd, J = 10.6 and 6.2 Hz, H_2-8a) and 3.87 (1H, dd, J = 10.6 and 6.6 Hz, H_2-8b), which were assigned to the oxygenated methylene; and four methyl signals at δ 1.88 (br s, H_3-10′), 0.97 (d, J = 6.7 Hz, H_3-9), 0.93 (d, J = 6.7 Hz, H_3-10), and 0.88 (t, J = 6.2 Hz, H_3-9′). The spectrum was completed by aliphatic multiplets at δ 1.45−1.21, 1.76, and 1.95, two doublets of doublets at δ 2.34 (H-2a) and 2.16 (H-2b), and a triplet at δ 2.61 (H_2-4′), all integrating for 20 protons. Analysis of the ^{1}H−^{1}H COSY experiment aided us to easily identify three spin systems, **a**, **b**, and **c** (Figure 2), which were connected by HMBC correlations. In particular, the −CO signal at δ 174.7 (C-1) showed cross-peaks with the methylene protons at δ 2.34/2.16 (H_2-2), thus defining one of the two terminals of the molecule, whereas the second −CO resonating at δ 167.1 (C-1′) was correlated with both the methylene at δ 3.96/3.87 (H_2-8) and the olefinic proton at δ 5.65 (H-2′), implying the connection of the two partial structures **a** and **c**. Diagnostic HMBC correlations were also observed between C-3′ (δ 160.8) and H_3-10′ (δ 1.88), H-2′ (δ 5.65), and H_2-4′ (δ 2.61), leading to structure **2**.

The geometry of the double bond at C-2′ was established as Z by both the carbon value of the vinyl methyl C-10′ (δ 25.2) and a NOE effect between H_3-10′ (δ 1.88) and H-2′ (δ 5.65). The configurations at C-3 and C-7 remain unassigned. Methylation of **2** gave the methyl ester **2a**, which was characterized by 2D-NMR experiments (see Experimental Section). The spectroscopic data were in agreement with the proposed structure. In particular, a long-range correlation between the introduced methoxy group

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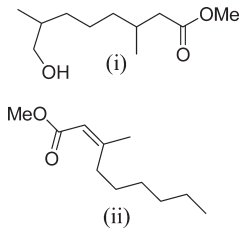

Figure 3. Methyl esters from the alkaline methanolysis of **2a**.

Table 3. NMR Spectroscopic Data[a] for Isofurospongin-4 (4)

position	δ_C mult.		δ_H (J in Hz)	HMBC[b]
1	142.4,	CH	7.33, br s	4
2	110.8,	CH	6.27, br s	1
3	124.9,	C		2, 4
4	138.4,	CH	7.20, br s	2
5	25.2,	CH_2	2.44, t (7.0)	2, 6
6	28.3,	CH_2	2.23, bq (7.0)	5, 7
7	123.5,	CH	5.16, br t (6.5)	6, 9
8	135.7,	C		7, 9, 10
9	15.6,	CH_3	1.58, s	7
10	39.6,	CH_2	1.97, t (7.8)	7, 9
11	26.5,	CH_2	2.05, m	10, 12
12	124.6,	CH	5.10, br t (6.3)	11, 14
13	135.4,	C		12, 14, 15
14	15.8,	CH_3	1.56, s	12, 15
15	39.0,	CH_2	2.02, m	12, 14
16	27.7,	CH_2	2.54, m	15, 17
17	143.1,	CH	5.95, br t (7.2)	16, 20
18	nd,	C		17
19	168.5,	C		17, OCH_3
20	33.5,	CH_2	2.33, m	17, 21
21	28.4,	CH_2	2.29, m	22
22	142.2,	CH	6.74, br t (6.8)	21, 24
23	127.7,	C		22, 24
24	12.0,	CH_3	1.79, s	22
25	169.3,	C		24
OCH_3	51.7,	CH_3	3.70, s	

[a] 600 MHz for ^{1}H NMR spectra and 75.47 MHz for ^{13}C NMR spectra ($CDCl_3$); assignments aided by COSY, HSQC, and HMBC. [b] HMBC correlations, optimized for 10 Hz, are from proton(s) stated to the indicated carbon.

(δ 3.66, OMe) and the terminal ester carbonyl carbon (δ 173.5, C-1) was observed in the HMBC spectrum of **2a**, along with all other expected correlations. To further confirm the proposed structure, compound **2a** was submitted to methanolysis by NaOMe solution to obtain a reaction mixture, which was analyzed by GC-MS. Diagnostic molecular ion peaks at m/z 202 and 184 due to methyl esters (i) and (ii) (Figure 3) produced by methanolysis were observed in the GC-MS spectrum.

Officinoic acid B (**3**) showed spectroscopic similarities with compound **2**. The molecular formula of **3**, $C_{22}H_{38}O_4$ as deduced by HRESIMS, contained an additional C_2H_2 unit with respect to acid **2**. The IR spectrum showed CO stretching bands at 1716 and 1667 cm^{-1} due to the presence of ester and terminal acid functions as in compound **2**. This was also supported by ^{13}C NMR resonances at δ 165.7 (CO, C-1′) and 173.8 (CO, C-1). The ^{1}H and ^{13}C NMR spectra of **3** indicated the presence of two trisubstituted double bonds, one of which is conjugated to an ester carbonyl, two vinyl methyls, two methyls linked to sp^3 carbons, an isolated methylene, and an oxygenated methine (Table 2). These data were consistent with a structure similar to compound **2** exhibiting different alkyl chains. The ^{1}H−^{1}H COSY and HMBC data (significant correlations are reported in Table 2) led us to formulate compound **3** as shown.

The geometries of the double bonds at C-3 and C-2′ were established as *Z*, *Z* by both the carbon values of the vinyl methyls C-11 and C-11′ and diagnostic NOE effects observed between these methyls and the respective olefin protons H-4 and H-2′. The configuration of C-9 remains unassigned. Analogous with **2**, compound **3** was converted into the corresponding methyl ester **3a**, which was characterized by 2D-NMR experiments (see Experimental Section). The subsequent methanolysis of **3a** under the same conditions as reported above for **2a** gave a reaction mixture, which was analyzed by GC-MS. Diagnostic molecular ion peaks at m/z 214 and 198 due to the two methyl esters produced in the reaction were observed in the spectrum, further confirming the proposed structure.

Compound **4** was isolated as a colorless, optically inactive oil and showed a positive Ehrlich reaction. The molecular formula $C_{26}H_{36}O_5$ was indicated by the sodiated molecular ion peak at m/z 451.2478 in the HRESIMS spectrum. The ^{1}H and ^{13}C NMR data of compound **4** closely resembled those of co-occurring furospongin-4 (**10**)[5a,d] (Table 3), which has the same molecular formula, suggesting that **4** is a linear furanosesterterpene with two oxidized methyl groups, analogous with **10**. Analysis of ^{1}H−^{1}H COSY and HSQC experiments confirmed this hypothesis, indicating that **4** differed from **10** only in the esterification site. Accordingly, the proton chemical shift value of H-17 was observed at δ 5.95 in **4** and at δ 6.01 in furospongin-4 (**10**). Diagnostic correlations in the HMBC spectrum of **4** between the carboxy carbon at δ 168.5 (C-19) and both the proton signals at δ 3.70 (3H, s, −OMe) and 5.95 (1H, br t, J = 7.2 Hz, H-17) inferred the esterification at C-19 rather than C-25. Compound **4** was thus named isofurospongin-4. The structure was definitively confirmed by conversion of **4** into the corresponding dimethyl ester **4a**. This compound was identical in all respects with **10a**, the derivative obtained by methylation of **10**. All ^{1}H and ^{13}C NMR resonances of the dimethyl ester **4a** (= **10a**) were assigned as reported in the Experimental Section.

Compounds **4**−**10** were tested for antibacterial and antifungal activities against *E. coli*, *Staphylococcus aureus*, and *Candida albicans*. Only furospongin-4 (**10**) showed weak activity against *S. aureus* at 100 μg/mL.

A very interesting biofilm induction activity was observed for selected terpene fractions containing tetrahydrofurospongin-2 (**6**) and dihydrofurospongin-2 (**7**). Both compounds were purified by HPLC and shown to be responsible for the observed activity (Figure 4). These data suggested that, even at a lower concentration (50 μg/mL), compounds **6** and **7** induced biofilm formation by *E. coli* PHL628 by a factor of 1.57 and 1.93, respectively. However, as the concentration increases, tetrahydrofurospongin-2 (**6**) becomes more efficient in inducing biofilm formation. Conversely, an increase of dihydrofurospongin-2 (**7**) concentration over 50 μg/mL had no additional effect. To our knowledge, this is the first case reported of sponge-produced molecules having bacterial biofilm induction properties. This phenomenon could be related to the symbiosis that marine

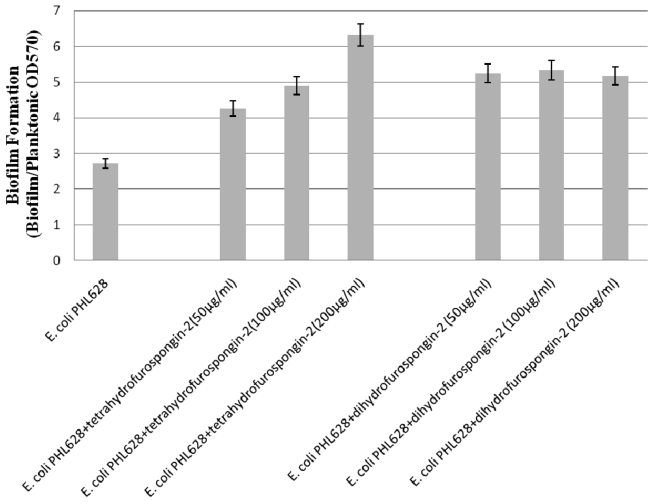

Figure 4. Biofilm formed by *Escherichia coli* PHL628 when incubated in the presence or absence of tetrahydrofurospongin-2 and dihydrofurospongin-2 in microtiter wells. The plate was incubated for 40 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD_{570}). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value is presented as the "biofilm formation" on the *y* axis. Bars represent means ± standard errors for six replicates.

organisms (i.e., algae and sponges) are able to form with some strains of bacteria that do not allow biofouling stratification on their surfaces.[12]

Indeed biofouling generally begins with the formation of a biochemical conditioning film onto which bacteria and other microorganisms colonize.[13] Closely following microbial fouling is the colonization by various eukaryotic organisms, including marine invertebrates and algae.[14] If the first bacterial biofilm does not allow the subsequent stratification, the sponge (or alga, or other marine organism) surface will not be contaminated by biofouling. Further studies will be necessary to understand the mechanism of biofilm induction.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Jasco DIP 370 digital polarimeter. IR spectra were measured on a Biorad FTS 155 FTIR spectrophotometer. 1D- and 2D-NMR spectra were recorded on a Bruker Avance-400 (400.13 MHz) and on a Bruker DRX-600 equipped with a TXI CryoProbe in $CDCl_3$ (δ values are referenced to $\underline{C}HCl_3$ at 7.26 ppm), and ^{13}C NMR spectra were recorded on a Bruker DPX-300 (75.47 MHz) (δ values are referenced to $\underline{C}DCl_3$, 77.0 ppm). HRESIMS was conducted on a Micromass Q-TOF micro. GC-MS was carried out on an ion-trap MS instrument in EI mode (70 eV) (Thermo, Polaris Q) connected with a GC system (Thermo, GCQ) by a 5% diphenyl (30 m × 0.25 mm × 0.25 um) column using helium as gas carrier. For HPLC, Waters 501 pumps with a refractometer detector were used with a reversed-phase column (Kromasil C-18, 5 μ, 250 × 4.60 mm, Phenomenex). TLC plates (Kieselgel 60 F_{254}) and silica gel powder (Kieselgel 60, 0.063−0.200 mm) were from Merck.

Biological Material. The sponge *Spongia officinalis* Linnaeus 1759 was collected in May 1999 off Mazara del Vallo along the Sicily coast by one of us (G.V.) at a depth of 80 m, immediately frozen, and transferred to ICB. The sponge was classified by R.v.S. A voucher specimen is stored at the Zoological Museum, University of Amsterdam (numbered as ZMAPOR 21294).

Extraction and Isolation Procedures. The frozen sponge *S. officinalis* (dry weight, 15.2 g) was chopped and then extracted exhaustively with Me_2CO (400 mL × 4) using ultrasound. After filtration and evaporation *in vacuo* of the organic solvent, the residue was subsequently extracted with Et_2O (200 mL × 4) and BuOH (100 mL × 4). The evaporation of the Et_2O and BuOH extracts gave two gummy residues (0.680 and 0.722 g, respectively). The Et_2O extract was analyzed by TLC. Two groups of molecules with different polarity were evidenced by positive reactions with the Ehrlich reagent: a nonpolar fraction at R_f 0.9−0.7 (light petroleum ether/Et_2O, 1:1) and a polar fraction at R_f 0.5−0.4 (light petroleum ether/Et_2O, 1:1). This extract was subjected to silica gel column chromatography using a gradient of light petroleum ether and Et_2O, then $CHCl_3$, and finally MeOH as eluents, to give anhydrofurospongin-1 (**5**) (25 mg), tetrahydrofurospongin-2 (**6**) (65 mg), dihydrofurospongin-2 (**7**) (40 mg), furospongin-1 (**8**) (140 mg), and a more polar fraction (350 mg), which consisted of a complex terpene mixture. This mixture was further fractionated by Si gel column chromatography, using a gradient of light petroleum ether and Et_2O as eluent, obtaining three fractions, A−C. The less polar fraction, A (90 mg), was purified by reversed-phase HPLC (MeOH/H_2O, 9:1) to give 7,8-epoxyfurospongin-1 (**1**) (1.2 mg), furospongolide (**9**) (1.8 mg), furospongin-4 (**10**) (1.6 mg), officinoic acid A (**2**) (0.8 mg), and officinoic acid B (**3**) (0.9 mg). Fraction B (87 mg) was purified by reversed-phase HPLC (MeOH/H_2O gradient), giving isofurospongin-4 (**4**) (2.5 mg). Fraction C (110 mg) was purified by reversed-phase HPLC (MeOH/H_2O, 95:5) to give 12α-deoxoscalarin (**11**) (0.9 mg), 16-deacetoxy-12-epi-scalarafuranacetate (**12**) (1.3 mg), and scalaradial (**13**) (1.0 mg). Tetrahydrofurospongin-2 (**6**) and dihydrofurospongin-2 (**7**) were further purified by reversed-phase HPLC (MeOH/H_2O, 95:5).

Furospongin-1 (***8***)*:* colorless oil; $[\alpha]_D$ +22 (*c* 1.2, $CHCl_3$), $[\alpha]_D$ lit.[5a] +8.8 (*c* 1.0, $CHCl_3$).

7,8-Epoxyfurospongin-1 (***1***)*:* colorless oil; R_f 0.45 (light petroleum ether/Et_2O, 1:1); $[\alpha]_D$ −12 (*c* 0.1, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 220 (2.82); IR (liquid film) ν_{max} 1620, 1575, 1160, 1020 cm^{-1}; ^1H and ^{13}C NMR data in Table 1; HRESIMS *m*/*z* 369.2040 $[M + Na]^+$ (calcd for $C_{21}H_{30}O_4Na$, 369.2042).

Officinoic acid A (***2***)*:* colorless oil; R_f 0.48 (light petroleum ether/Et_2O, 1:1); $[\alpha]_D$ +3.2 (*c* 0.08, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 215 (2.43); IR (liquid film) ν_{max} 1710, 1651 cm^{-1}; ^1H and ^{13}C NMR data in Table 2; HRESIMS *m*/*z* 363.2498 $[M + Na]^+$ (calcd for $C_{20}H_{36}O_4Na$, 363.2511).

Officinoic acid B (***3***)*:* colorless oil; R_f 0.50 (light petroleum ether/Et_2O, 1:1); $[\alpha]_D$ −5.7 (*c* 0.09, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ε) 218 (2.72); IR (liquid film) ν_{max} 1710, 1667 cm^{-1}; ^1H and ^{13}C NMR data in Table 2; HRESIMS *m*/*z* 389.2679 $[M + Na]^+$ (calcd for $C_{22}H_{38}O_4Na$, 389.2668).

Isofurospongin-4 (***4***)*:* colorless oil, R_f 0.42 (light petroleum ether/Et_2O, 1:1); UV ($CHCl_3$) λ_{max} (log ε) 208 (3.12); IR (liquid film) ν_{max} 1705, 1620 cm^{-1}; ^1H and ^{13}C NMR data in Table 3; HRESIMS *m*/*z* 451.2478 $[M + Na]^+$ (calcd for $C_{26}H_{36}O_5Na$, 451.2460).

Preparation of Methylated Compounds 2a, 3a, 4a, and 10a. A 1 mL amount of diazomethane in Et_2O was added to 0.8 mg of compound **10**. After 0.5 h the Et_2O was evaporated *in vacuo*, and compound **10a** was quantitatively obtained. The same procedures were repeated for 0.8 mg of compound **2**, 0.9 mg of compound **3**, and 1 mg of compound **4** to afford **2a**, **3a**, and **4a**, respectively.

Officinoic acid A methyl ester (***2a***)*:* colorless oil; ^1H NMR ($CDCl_3$, 600 MHz) δ 5.65 (1H, br s, H-2′), 3.85 (1H, dd, *J* = 10.6, 5.9 Hz, H-8a), 3.87 (1H, dd, *J* = 10.6, 6.6 Hz, H-8b), 3.66 (3H, s, OCH_3), 2.61 (2H, t, *J* = 7.3 Hz, H_2-4′), 2.30 (1H, dd, *J* = 14.6, 6.2 Hz, H-2a), 2.13 (1H, dd, *J* = 14.6, 7.6 Hz, H-2b), 1.93 (1H, m, H-3), 1.88 (3H, s, H_3-10′), 1.78 (1H, m, H-7), 1.45 (2H, m, H_2-5′), 1.40 (2H, m, H_2-5), 1.32 (2H, m, H_2-6′), 1.31 (2H, m, H_2-7′), 1.29 (2H, m, H_2-4), 1.29 (2H, m, H_2-8′), 1.19 (2H, m, H_2-6), 0.93 (3H, d, *J* = 6.7 Hz, H_3-9), 0.92 (3H, d, *J* = 6.6 Hz, H_3-10),

0.88 (3H, t, J = 7.0 Hz, H_3-9′). ^{13}C NMR ($CDCl_3$): δ 173.5 (C, C-1), 165.9 (C, C-1′), 160.9 (C, C-3′), 116.0 (CH, C-2′), 68.4 (CH_2, C-8), 51.4 (OCH_3), 41.7 (CH_2,C-2), 33.8 (CH_2, C-4′), 32.7 (CH, C-7), 31.8 (CH_2, C-7′), 30.8 (CH_2, C-6), 30.7 (CH, C-3), 30.6 (CH_2, C-5), 29.7 (CH_2, C-6′), 29.7 (CH_2, C-4), 28.3 (CH_2, C-5′), 25.4 (CH_3, C-10′), 22.6 (CH_2, C-8′), 19.6 (CH_3, C-9), 16.8 (CH_3, C-10), 14.1 (CH_3, C-9′); HRESIMS m/z 377.2671 $[M + Na]^+$ (calcd for $C_{21}H_{38}O_4Na$, 377.2668).

*Officinoic acid B methyl ester (**3a**):* colorless oil; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 5.62 (1H, br s, H-2′), 5.33 (1H, br t, J = 7.3 Hz, H-4), 4.91 (1H, dq, J = 6.3, 5.5 Hz, H-9), 3.67 (3H, s, OCH_3), 3.04 (2H, br s, H_2-2), 2.61 (2H, t, J = 7.0 Hz, H_2-4′), 2.01 (2H, m, H_2-5), 1.87 (3H, s, H_3-11′), 1.76 (3H, br s, H_3-11), 1.60 (1H, m, H-8a), 1.49 (H, m, H-8b), 1.48 (2H, m, H_2-5′), 1.33 (2H, m, H_2-6), 1.30 (2H, m, H_2-7), 1.30 (2H, m, H_2-7′), 1.30 (2H, m, H_2-8′), 1.29 (2H, m, H_2-6′), 1.28 (2H, m, H_2-9′), 1.21 (3H, d, J = 6.2 Hz, H_3-10), 0.89 (3H, t, J = 6.6 Hz, H_3-10′). ^{13}C NMR ($CDCl_3$): δ 171.8 (C, C-1), 165.7 (C, C-1′), 160.1 (C, C-3′), 128.9 (CH, C-4), 128.5 (C, C-3), 116.3 (CH, C-2′), 69.7 (CH, C-9), 51.6 (OCH_3), 37.1 (CH_2, C-2), 35.9 (CH_2, C-8), 33.3 (CH_2, C-4′), 31.7 (CH, C-8′), 29.8 (CH_2, C-7), 29.3 (CH_2, C-6), 29.3 (CH_2, C-7′), 29.2 (CH_2, C-6′), 28.1 (CH_2, C-5), 27.8 (CH_2, C-5′), 24.9 (CH_3, C-11′), 23.8 (CH_3, C-11), 22.4 (CH_2, C-9′), 20.0 (CH_3, C-10), 14.1 (CH_3, C-10′); HRESIMS m/z 403.2827 $[M + Na]^+$ (calcd for $C_{23}H_{40}O_4Na$, 403.2824).

*Methyl isofurospongin-4 (**4a**):* colorless oil; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.32 (1H, br s, H-1), 7.21 (1H, br s, H-4), 6.73 (1H, br t, J = 7.3 Hz, H-22), 6.28 (1H, br s, H-2), 5.91 (1H, br t, J = 7.3 Hz, H-17), 5.16 (1H, br t, J = 6.6 Hz, H-7), 5.12 (1H, br t, J = 6.3 Hz, H-12), 3.74 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 2.54 (2H, q, J = 7.3 Hz, H_2-16), 2.44 (2H, t, J = 7.0 Hz, H_2-5), 2.36 (2H, t, J = 7.3 Hz, H_2-20), 2.30 (2H, t, J = 7.4 Hz, H_2-21), 2.25 (2H, br q, J = 7.0 Hz, H_2-6), 2.07 (2H, m, H_2-15), 2.04 (2H, m, H_2-11), 1.98 (2H, t, H_2-10), 1.81 (3H, s, H_3-24), 1.59 (3H, br s, H_3-9), 1.59 (3H, br s, H_3-14). ^{13}C NMR ($CDCl_3$): δ 168.3 (C, C-19), 168.3 (C, C-25), 143.3 (CH, C-17), 142.3 (CH, C-1), 141.0 (CH, C-22), 138.5 (CH, C-4), 135.6 (C, C-8), 135.4 (C, C-13), 128.1 (C, C-23), 124.9 (CH, C-12), 124.7 (C, C-3), 123.5 (CH, C-7), 111.0 (CH, C-2), 51.5 (OCH_3), 51.5 (OCH_3), 39.6 (CH_2, C-10), 39.0 (CH_2, C-15), 33.4 (CH_2, C-20), 28.5 (CH_2, C-21), 28.4 (CH_2, C-6), 27.8 (CH_2, C-16), 26.6 (CH_2, C-11), 25.2 (CH_2, C-5), 15.9 (CH_3, C-9), 15.9 (CH_3, C-14), 12.3 (CH_3, C-24); HRESIMS m/z 465.2626 $[M + Na]^+$ (calcd for $C_{27}H_{38}O_5Na$, 465.2617).

Preparation of MTPA Esters of 7,8-Epoxyfurospongin-1 (1). *7,8-Epoxyfurospongin-1 (S-MTPA ester) (**1a**).* The *S*-MTPA ester was prepared by treating 0.6 mg of **1** with 0.005 mL of *R*-(−)-MTPA chloride in dry CH_2Cl_2 (0.5 mL) with a catalytic amount of DMAP under stirring for 16 h at room temperature. The ester was purified by chromatography in a Pasteur pipet (SiO_2, $CHCl_3$). Selected ^{1}H NMR values ($CDCl_3$, 600 MHz) δ 5.2090 (H-11), 2.6921 (H-7), 2.5384 (H_2-5), 2.3583 (H_2-17), 2.0721 (H_2-10), 1.7282 (H_2-6), 1.4900 (H_2-12), 1.1914 (H_3-9), 0.8978 (H_3-14); ESIMS m/z 562 $[M + Na]^+$.

*7,8-Epoxyfurospongin-1 (R-MTPA ester) (**1b**).* The *R*-MTPA ester was prepared by treating 0.6 mg of **1** with 0.005 mL of *S*-(+)-MTPA chloride in dry CH_2Cl_2 (0.5 mL) with a catalytic amount of DMAP under stirring for 16 h at room temperature. The ester was purified by chromatography in a Pasteur pipet (SiO_2, $CHCl_3$). Selected ^{1}H NMR values ($CDCl_3$, 600 MHz) δ 5.2600 (H-11), 2.7490 (H-7), 2.5732 (H_2-5), 2.2714 (H_2-17), 2.1380 (H_2-10), 1.7720 (H_2-6), 1.4000 (H_2-12), 1.2810 (H_3-9), 0.8265 (H_3-14); ESIMS m/z 562 $[M + Na]^+$.

Alkaline Methanolysis of Officinoic Acid A and Officinoic Acid B Methyl Esters (2a and 3a). Compounds **2a** and **3a** were treated with 1 mL of a methanolic solution of sodium methoxide (3 M). The reaction mixture was stirred overnight at room temperature. After evaporation, the mixture was filtered over Si gel in a Pasteur pipet, the products were eluted with Et_2O, and the crude filtrate was analyzed by GC-MS.

Biological Assays. The antifungal assay was performed by the broth macrodilution method following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) document M27-P.[15,16] The antibacterial assay was performed by using the same method as the antifungal test, differing only in the assay medium (Luria−Bertani medium: 10 g/L Bactotryptone, 5 g/L Bactoyeast, and 10 g/L NaCl, pH 7.5) and in the incubation temperature (37 °C for 24 h).

Biofilm Assay. The assay method used was a modified version of that described by Djordjevic et al.[17] *Escherichia coli* PHL628 strain was grown overnight at 37 °C in 5 mL of defined minimal medium, M63, containing kanamycin (50 μg/mL). Overnight cultures were then refreshed again in M63 medium, incubated at 37 °C for 5 to 6 h, and vortexed; then 200 μL of inocula was introduced in the 96-well polystyrene microtiter plate with an initial turbidity at 600 nm of 0.05 in the presence or absence of compound to be tested. The microtiter plate was then left at 30 °C for 40 h under static conditions.

To correlate biofilm formation with planktonic cell growth in each well, the planktonic cell fraction was transferred to a new microtiter plate, and the OD_{570} was measured using a microtiter plate reader (Multiscan Spectrum, Thermo Electron Corporation). To assay biofilm formation, the remaining volume from the incubated microtiter plate was removed and the wells were washed five times with sterile distilled H_2O to remove loosely associated bacteria. Plates were air-dried for 45 min, and each well was stained with 200 μL of 1% crystal violet solution for 45 min. After staining, the plates were washed with sterile distilled H_2O five times. The quantitative analysis of biofilm production was performed by adding 200 μL of EtOH/acetone solution (4:1) to destain the wells. The level (OD) of the crystal violet present in the destaining solution was measured at 570 nm. Biofilm formation was calculated by dividing the total biofilm by the bacterial growth. Six replicate wells were made for each experimental parameter, and each data point was an average from the six replicate wells.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ DEDICATION

Dedicated to Dr. Guido Cimino on his 70th birthday.

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MICROBIAL CELL FACTORIES

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Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of *Bacillus licheniformis*

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Abstract

Background: Secondary metabolites ranging from furanone to exo-polysaccharides have been suggested to have anti-biofilm activity in various recent studies. Among these, *Escherichia coli* group II capsular polysaccharides were shown to inhibit biofilm formation of a wide range of organisms and more recently marine *Vibrio* sp. were found to secrete complex exopolysaccharides having the potential for broad-spectrum biofilm inhibition and disruption.

Results: In this study we report that a newly identified ca. 1800 kDa polysaccharide having simple monomeric units of α-D-galactopyranosyl-(1→2)-glycerol-phosphate exerts an anti-biofilm activity against a number of both pathogenic and non-pathogenic strains without bactericidal effects. This polysaccharide was extracted from a *Bacillus licheniformis* strain associated with the marine organism *Spongia officinalis*. The mechanism of action of this compound is most likely independent from quorum sensing, as its structure is unrelated to any of the so far known quorum sensing molecules. In our experiments we also found that treatment of abiotic surfaces with our polysaccharide reduced the initial adhesion and biofilm development of strains such as *Escherichia coli* PHL628 and *Pseudomonas fluorescens*.

Conclusion: The polysaccharide isolated from sponge-associated *B. licheniformis* has several features that provide a tool for better exploration of novel anti-biofilm compounds. Inhibiting biofilm formation of a wide range of bacteria without affecting their growth appears to represent a special feature of the polysaccharide described in this report. Further research on such surface-active compounds might help developing new classes of anti-biofilm molecules with broad spectrum activity and more in general will allow exploring of new functions for bacterial polysaccharides in the environment.

Background

Most species of bacteria prefer biofilm as the most common means of growth in the environment and this kind of bacterial socialization has recently been described as a very successful form of life on earth [1]. Although they can have considerable advantages in terms of self-protection for the microbial community involved or to develop *in situ* bioremediation systems [2], biofilms have great negative impacts on the world's economy and pose serious problems to industry, marine transportation, public health and medicine due to increased resistance to antibiotics and chemical biocides, increased rates of genetic exchange, altered biodegradability and increased production of secondary metabolites [3-8]. Therefore, based on the above reasons, development of anti-biofilm strategies is of major concern.

The administration of antimicrobial agents and biocides in the local sites to some extent has been a useful approach to get rid of biofilms [9], but prolonged persistence of these compounds in the environment could induce toxicity towards non-target organisms and resistance among microorganisms within biofilms. This aspect has led to the development of more environment friendly compounds to combat with the issue. It has been found that many organisms in the marine areas maintain a clean surface. Most of the marine invertebrates have developed unique ways to combat against potential invaders, predators or other competitors [10] especially through the production of specific compounds toward biofilm-forming microorganisms [11]. Nowadays, it is

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hypothesized that bioactive compounds previously thought to be produced from marine invertebrates might be produced by the associated microorganisms instead. Various natural compounds from marine bacteria, alone or in association with other invertebrates, are emerging as potential sources for novel metabolites [12] and have been screened to validate anti-biofilm activity. The quorum sensing antagonist (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (furanone) from the marine alga *Delisea pulchra* inhibits biofilm formation in *E. coli* without inhibiting its growth [13]. The metabolites of a marine actinomycete strain A66 inhibit biofilm formation by *Vibrio* in marine ecosystem [12]. Extracts from coral associated *Bacillus horikoshii* [14] and actinomycetes [15] inhibit biofilm formation of *Streptococcus pyogenes*. The exoproducts of marine *Pseudoalteromonas* impair biofilm formation by a wide range of pathogenic strains [16]. Most recently, exo-polysaccharides from the marine bacterium *Vibrio* sp. QY101 were shown to control biofilm-associated infections [17].

Compounds secreted or extracted from marine microorganisms having anti-biofilm activity range from furanone to complex polysaccharide. Although bacterial extracellular polysaccharides synthesized and secreted by a wide range of bacteria from various environments have been proven to be involved in pathogenicity [18], promotion of adherence to surfaces [19-21] and biofilm formation [22,23], recent findings suggest that some polysaccharides secreted from marine and non marine organisms also possess the ability to negatively regulate biofilm formation [17,24-27].

In this study, we show that an exo-polysaccharide purified from the culture supernatant of bacteria associated to a marine sponge (*Spongia officinalis*) is able to inhibit biofilm formation without affecting the growth of the tested strains. Phylogenetic analysis by 16S rRNA gene sequencing identified the sponge-associated bacterium as *Bacillus licheniformis*. The mechanisms behind the anti-biofilm effect of the secreted exo-polysaccharide were preliminarily investigated.

Results

Bacillus licheniformis culture supernatant inhibits biofilm formation by *Escherichia coli* PHL628

Starting from a *Spongia officinalis* sample, it has been possible to distinguish, among one hundred colonies of sponge-associated bacteria, ten different kinds in terms of shape, size and pigmentation. They were screened for production of bioactive anti-biofilm metabolites. One colony for each phenotype was grown till stationary phase and the filtered cell-free supernatants obtained were used at a concentration of 3% (v/v) against a stationary culture of the indicator strain *E. coli* PHL628 (Figure 1). Supernatants derived from strains SP1 and SP3 showed a strong anti-biofilm activity (65% and 50% reduction, respectively). SP1 was chosen to study the nature of the biofilm inhibition mechanism. Sequencing of the 16S RNA revealed that the SP1 gene showed 99% similarity with *Bacillus licheniformis*.

Isolation and purification of active compounds

The active fraction of SP1 cell free supernatant was initially found to be of polysaccharidic composition. Preliminary spectroscopic investigations indicated the presence of a compound with a simple primary structure; the ^{1}H and ^{13}C NMR spectra suggested that the polymer was composed by a regular-repeating unit; the monosaccharide was identified as an acetylated O-methyl glycoside derivative and the compositional analysis was completed by the methylation data which indicated the presence of 4-substituted galactose; in fact the sample was methylated with iodomethane, hydrolized with 2 M triofluoroacetic acid (100°C, 2 h), the carbonyl was reduced by $NaBD_4$, acetylated with acetic anhydride and pyridine, and analyzed by GC-MS. The molecular mass of the polysaccharidic molecule was estimated to be approximately 1800 kDa by gel filtration on a Sepharose CL6B. In TOCSY, DEPT-HSQC, and HSQC-TOCSY experiments, additional signals of a -CHO- and two -CH_2 O- spin system proved the presence of not only a galactose residue but also of a glycerol residue (Gro); the relatively deshielded value for the glycerol methylene carbons at 65.6 and 65.4 ppm was consistent with a phosphate substitution at C1 of glycerol. ^{31}P-NMR spectrum confirms the presence of a phosphodiester group.

The position of the phosphate group between the α-D-galactopyranosyl and the glycerol residue was unambiguously confirmed with 2D ^{1}H ^{31}P-HSQC experiments. In fact, correlations between the ^{31}P resonance and H4 (3.827 ppm) of galactose were observed. This fact established the connectivity of the phosphate group to the respective carbon atoms. It follows that the repeating unit contains the phosphate diester fragment. Galactose was present as pyranose ring, as indicated by ^{1}H- and ^{13}C-NMR chemical shifts and by the HMBC spectrums that showed some typical intra-residual scalar connectivities between H/C (Table 1). The connection between galactose and glycerol into repeating unit was determined using HMBC and NOE effects. The anomeric site (99.47 and 5.071 ppm) of galactose presented long-range correlations with glycerol C2' (70.76 ppm) and H2' (4.120 ppm), and allowed the localization of galactose binding at C2' of glycerol. NOE contacts of anomeric proton at 5.071 ppm with the signal at 3.839 ppm (Gro H23', table 1) confirmed this hypothesis.

Thus, the polysaccharide is composed of α-D-galactopyranosyl-(1→2)-glycerol-phosphate monomeric units (Figure 2).

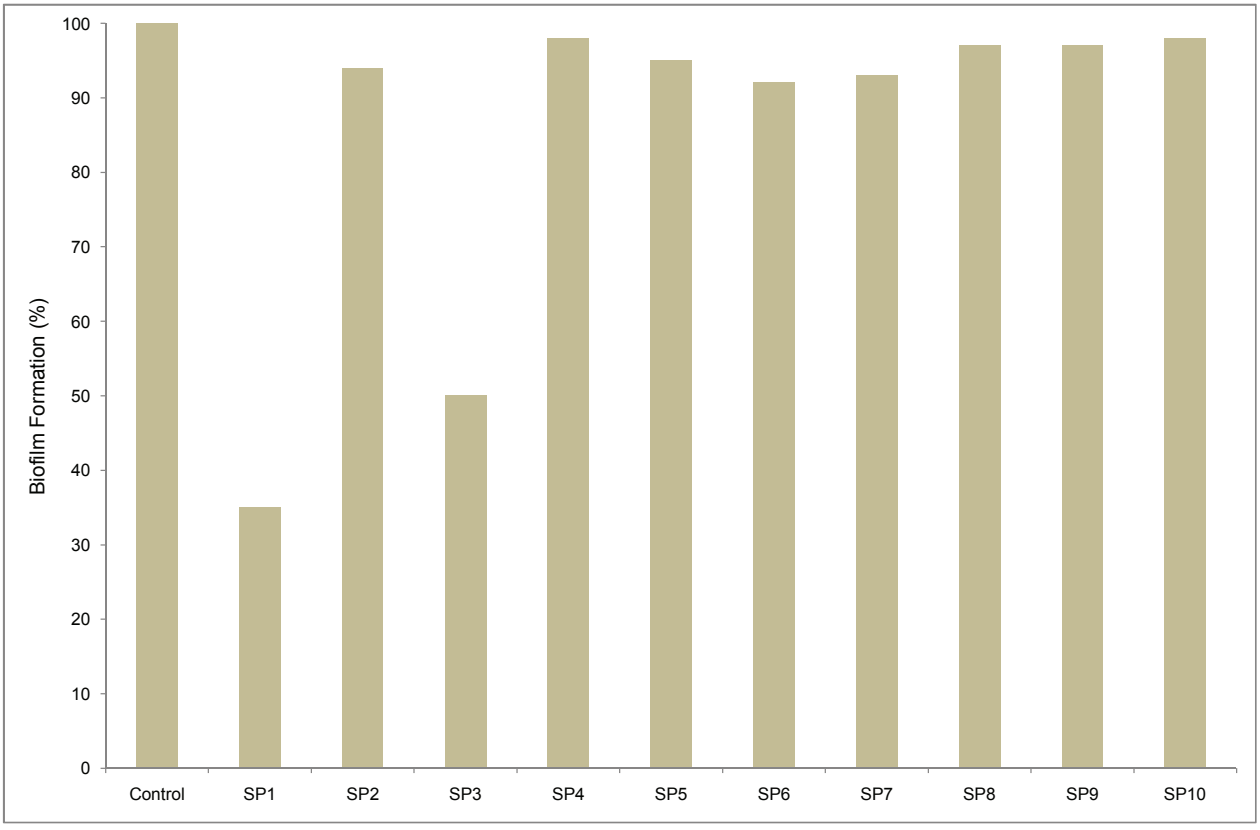

Figure 1 Anti-biofilm activity of supernatants from different strains (SP1-SP10) associated with *Spongia officinalis*. Biofilms of *Escherichia coli* PHL628 were allowed to develop in the presence of supernatants (3% v/v) from marine sponge-associated isolates in 96 well microtiter well. The plate was incubated at 30°C for 36 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD_{570}). The absorbance was used to calculate the "biofilm formation" on the *y axis*. × *axis* represents cell free supernatants from different *Spongia officinalis* isolates. The 100% is represented by *E. coli* PHL628 produced biofilm.

Table 1 ^{1}H, ^{13}C and ^{31}P NMR chemical shift of polysaccharide(p.p.m). Spectra in D_2O were measured at 27°C and referenced to internal sodium 3-(trimethylsilyl)-(2,2,3,3-$^{2}H_4$) propionate (δ_H 0.00), internal methanol (δ_C 49.00) and to external aq. 85% (v/v) phosphoric acid (δ_P 0.00)

Residue	Nucleus	1	2	3	4	5	6
→ 4)-α-D-Galp-(1 →							
	^{1}H	5.071 ^{H3Gro} (3.7 Hz)[a]	3.690	3.784	*3.827* ^{C6,4Gal}	*3.917* ^{C3Gal}	3.671 ^{H1Gal}
	^{13}C	*99.47* ^{H1Gro}	69.37	69.95	*78.32* ^{H1Gal} (7.8 Hz)[b]	70.19	*62.18* ^{H5Gal}
Gro-1-P-(O →							
	^{1}H	*3.865* ^{C4Gal} -3.906	*4.120* $^{C3,\ 5Gro}$	3.839-3.770			
	^{13}C	*65.63** ^{H1Gro} (4 Hz)[a] -65.41* (4.5)[a]	*70.76* ^{H1Gal} (7.9 Hz)[c]	67.15 (~2 Hz)[d]			
	^{31}P	1.269					

*diastereotopic carbons; [a] $^{3}J_{H1,\ H\ 2}$; [b] $^{2}J_{C\text{-}P}$; [c] $^{3}J_{C\text{-}P}$; [d] $^{4}J_{C\text{-}P}$; in italics, the signals showing C-H long-range correlations with the positions in superscripts; underlined are the NOE contacts with positions in superscripts.

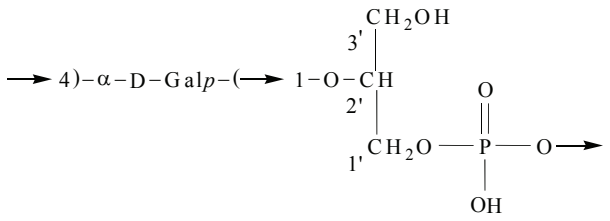

Figure 2 Repeating unit of the bacterial polysaccharide having anti-biofilm activity.

The anti-biofilm activity does not result from reducing *E. coli* and *P. fluorescens* growth

In order to check whether the anti-biofilm activity of the sponge-associated SP1 strain is dependent on the concentration used in the microtiter plate assay, the cell free supernatant from this strain was tested against biofilm formation by two organisms, *E. coli* PHL628 and *Pseudomonas fluorescens*. The results of Figure 3 clearly show that the anti-biofilm activity raises as the concentration of the supernatant increases. The anti-biofilm activity of the SP1 supernatant against the two test strains was comparable and perhaps slightly higher for *E. coli* PHL628, as in the presence of 5% (v/v) supernatant, inhibition was about 89% and 80% on biofilm formation by *E. coli* PHL 628 (Figure 3A) and *Pseudomonas fluorescens*, respectively (Figure 3B).

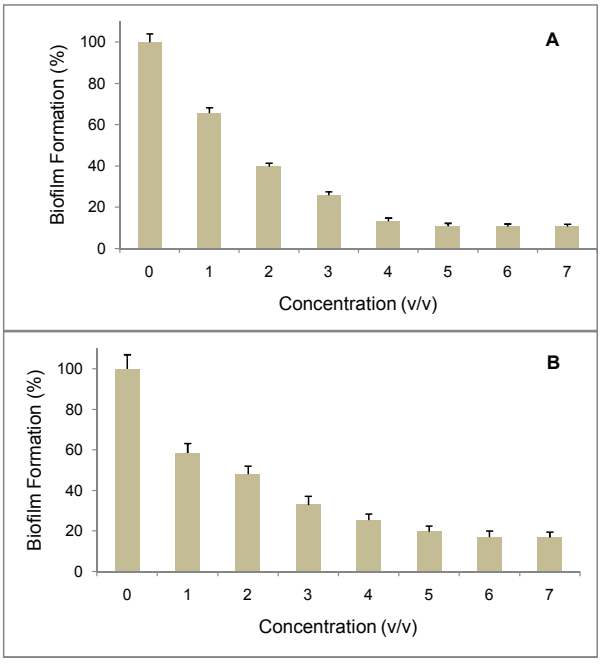

Figure 3 Anti-biofilm activity is concentration-dependent. Stationary cells of *E. coli* PHL628 (A) or *P. fluorescens* (B) were incubated along with the SP1 supernatant at different concentrations in 96-well microtiter plate. The plate was incubated at 30°C for 36 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD_{570}). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value was used to calculate the "biofilm formation" on the *y axis*. × *axis* represents the concentration of supernatant used in the wells. Bars represent means ± standard errors for six replicates.

To evaluate whether the anti-biofilm effect of cell-free supernatant from sponge-associated *B. licheniformis* was related to reduction of growth rate of the target strains, growth curves of both strains were measured in presence and absence of 5% (v/v) supernatant. The resulting growth rates were found to be the same in the two conditions for both *E. coli* PHL628 (0.51 ± 0.02 h^{-1}) and *P. fluorescens* (0.69 ± 0.02 h^{-1}), clearly indicating that the supernatant has no bactericidal activity against the cells of biofilm-producing *E. coli* PHL628 or *P. fluorescens*. These data were further confirmed by the disc diffusion assay. No inhibition halo surrounding the discs was observed, thereby indicating that the supernatant has no bacteriostatic or bactericidal activity against *E. coli* PHL628 and *P. fluorescens*.

The efficiency of the sponge-associated SP1 supernatant for anti-biofilm activity was evaluated also by microscopic visualization. This approach confirmed that the inhibitory effect of the supernatant on biofilm formation increases with the increase of its concentration. Ten-fold concentrated supernatant completely inhibited biofilm formation by *E. coli* PHL628. Less concentrated supernatant also showed significant reduction of biofilm formation as compared to the control (Figure 4A). Very similar effects were observed with *P. fluorescens* (Figure 4B).

Inhibitory effect of the supernatant on various strains

To evaluate further the inhibitory effect of the SP1 supernatant on biofilm development, multiple strains regardless of pathogenicity were tested (Figure 5). Among the strains, 5 out of 10 appeared to be more than 50% inhibited in their biofilm development by the SP1 supernatant. Very interestingly, in the case of *Staphylococcus aureus*, the inhibition was almost 90%. Among the four *Bacillus* species, *B. amyloliquefaciens* was the most affected one, whereas *B. pumilis* and *B. cereus* were less affected in the inhibition of biofilm development. Not a single strain was stimulated or unaffected in biofilm development by the supernatant.

Preliminary characterization of the bio-active component of SP1 supernatant

The SP1 cell free supernatant gradually loses its efficiency in decreasing biofilm formation after its pretreatment at temperatures ranging from 50°C to 80°C. When the supernatant was treated at 50°C, the inhibitory activity towards *E. coli* PHL628 remained 100%, but at 60°C it started to decrease (95%). Treatment at 70°C

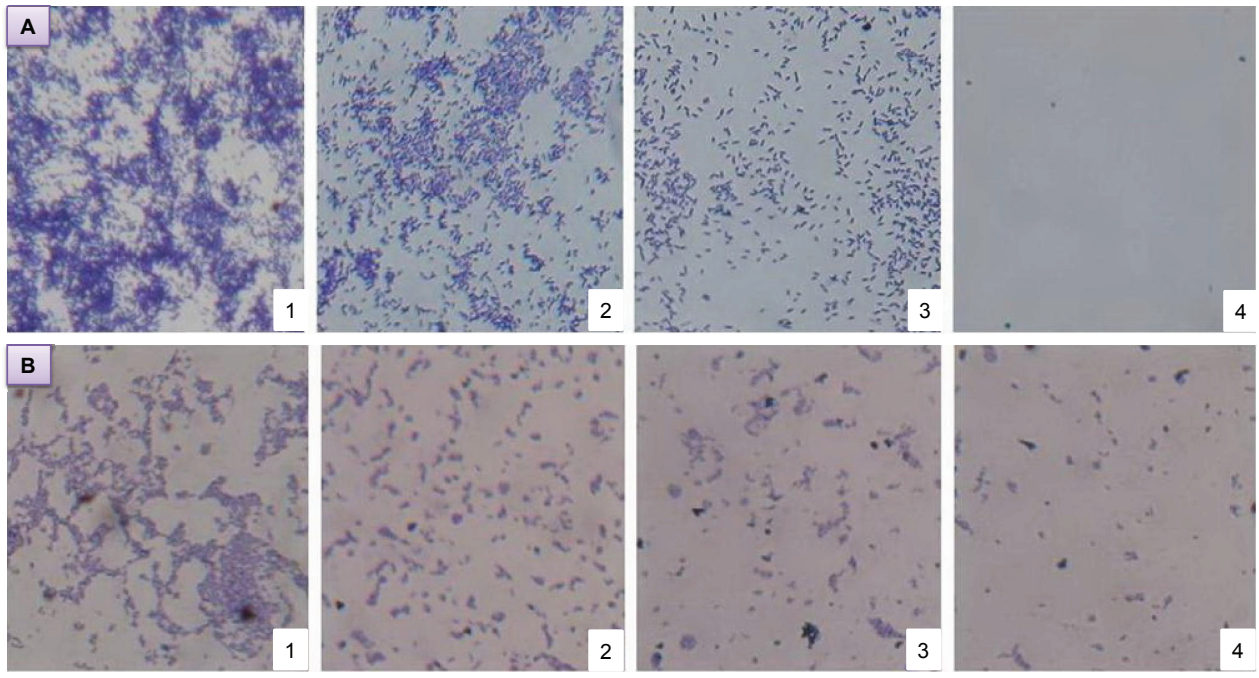

Figure 4 Microscope observation of biofilm inhibition. Biofilm inhibition of *E. coli* PHL628 (A) and *Pseudomonas fluorescens* (B) on glass cover slip under a phase-contrast microscope at a magnification of 40X. Bacterial cells were incubated with (1) 1X SP1 supernatant, (2) 2 × SP1 concentrated supernatant, (3) 5 × SP1 concentrated supernatant, (4) 10 × SP1 concentrated supernatant. No difference in biofilm production was observed in the presence of 1X, 2X, 5X and 10X $M63K_{10}$ sterile medium (not shown).

and 80°C, resulted in 41% and 29% of the anti-biofilm activity respectively. At 90°C the inhibitory activity was completely lost (data not shown).

To preliminarily characterize the mechanism of action of the SP1 supernatant, this was added to bacterial cells together with the quorum sensing signals obtained from two days supernatant of an *E. coli* PHL628 culture in order to understand if there is a competition for the quorum sensing receptor. The use of the two supernatants together had almost the same effect on biofilm inhibition as the SP1 alone (data not shown).

To analyze whether inhibition of biofilm production is related to reduced adherence of target cells to surfaces, we tested (see Methods) the effects of SP1 supernatant on the degree of cell surface hydrophobicity of *E. coli* PHL628 and *P. fluorescens*. As shown in Figure 6, the supernatant inhibits significantly the surface hydrophobicity of *E. coli* and to a lesser extent also that of *P. fluorescens*.

Pre-coating with SP1 supernatant inhibits initial attachment to the abiotic surface

The polysaccharide present in the SP1 supernatant might modify the abiotic surface in such a way that there might be a reduction or inhibition of irreversible attachment of the biofilm forming bacteria to an inanimate object. We tested this hypothesis by analyzing whether there is an effect on biofilm production by *E. coli* PHL628 if the polystyrene wells of the microtiter plate are pre-coated with SP1 supernatant. We observed that after 36 h, while biofilm formation was inhibited by 75% in the un-coated wells and in presence of supernatant, in the pre-coated wells the biofilm assay performed an inhibition of 92.5% (Figure 7). In addition, to evaluate further the mechanism of action in the initial attachment stage of biofilm development, the supernatant was added in the already formed biofilm. The effects were found to be much lower compared to that of the initial addition or pre-coating of the supernatant in the microtiter wells. A possible conclusion of this experiment is that the supernatant modifies the target surface in a way that prevents biofilm formation and that the initial attachment step is most important for biofilms production, at least by the organisms studied in this work.

Discussion

Marine biota is a potential source for the isolation of novel anti-biofilm compounds [12]. It has been estimated that among all the microbes isolated from marine invertebrates, especially sponge associated, *Bacillus* species are the most frequently found members so far [28]. Therefore the identification, in the present study, of a sponge-associated *Bacillus licheniformis* having anti-biofilm activity is not surprising. Our study demonstrates

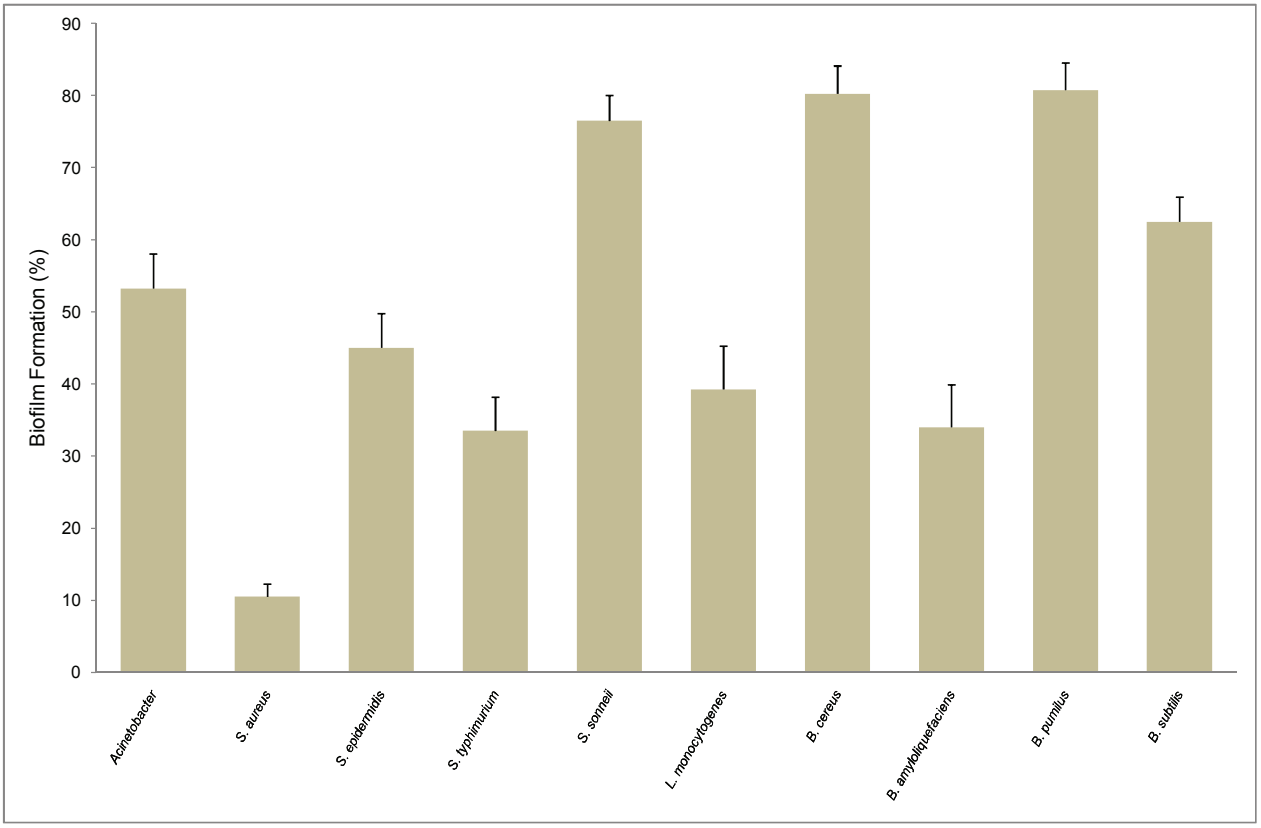

Figure 5 Inhibitory effect of the SP1 supernatant over a range of Gram-positive and Gram-negative bacteria. Biofilms of various Gram-positive and Gram-negative bacteria were developed in the presence or absence of the SP1 supernatant (5% V/v) in 96-well microtiter plate. The plate was incubated at 30°C for 36 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD_{570}). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value used to calculate the "biofilm formation" on the *y axis*. The various Gram-positive and Gram-negative bacteria used in the wells are listed on *X axis*. Bars indicate means ± standard errors for six replicates.

the occurrence of anti-biofilm activity of a previously uncharacterized polymeric polysaccharide having monomeric structure of galactose-glycerol-phosphate. To our knowledge, no literature has ever reported the finding of such a bioactive compound from marine or other sources.

We found that the polysaccharide is secreted in the culture supernatant by the sponge-associated *B. licheniformis* and its addition to a range of Gram-positive and Gram-negative bacteria results in negative effect on their biofilm development. This broad spectrum of anti-biofilm activity might help *B. licheniformis* during a competitive edge in the marine environment to establish itself on the surface of host sponges and critically influence the development of unique bacterial community.

It has been previously reported that bacterial extracellular polysaccharides can be involved both in biofilm and anti-biofilm activities. For example EPSs from *V. cholera* containing the neutral sugars glucose and galactose are important architectural components of its biofilm [29-31]. On the other hand, EPSs from *E. coli* (group II capsular polysaccharide) [26], *V. vulnificus* (capsular polysaccharide) [32], *P. aeruginosa* (mainly extracellular polysaccharide) [27,33] and marine bacterium *Vibrio* sp. QY101 (exopolysaccharide) [17] display selective or broad spectrum anti-biofilm activity. However, the potentiality of the polysaccharide described in this study over a wide range of pathogenic and non pathogenic organisms suggests that the compound might be a powerful alternative among the previously identified polysaccharides in multispecies biofilm context.

Based on the findings, we hypothesize that our polysaccharide might interfere with the cell-surface influencing cell-cell interactions, which is the pre-requisite for biofilm development [34], or with other steps of biofilm assembling. It has been reported in other cases that polysaccharides can produce anti-adherence effects between microorganisms and surfaces [35]. The *E. coli* group II CPS and exo-polysaccharides of marine *Vibrio* sp. were reported to inhibit biofilm formation not only

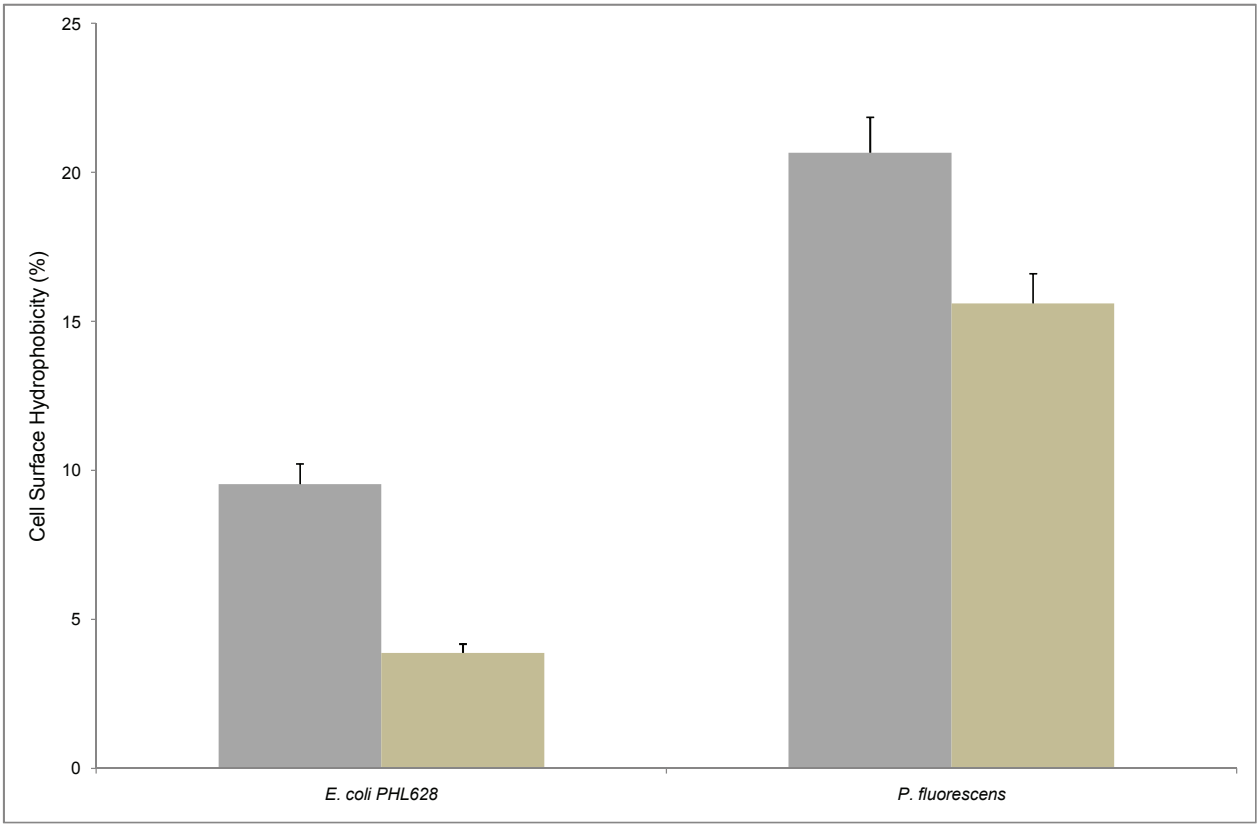

Figure 6 Cell surface hydrophobicity (CSH) assay for *E. coli* PHL628 and *P. fluorescens*. *E. coli* PHL628 and *P. fluorescens* were grown in minimal medium $M63K_{10}$ and M63, respectively, in the presence (light tan bars) and absence (gray bars) of SP1 supernatant. Bars represent means ± standard errors for six replicates.

by weakening cell-surface contacts but also by reducing cell-cell interactions or disrupting the interactions of cell-surfaces and cell-cell [26,17]. In all the previously described polysaccharides having anti-adherence property, highly anionic nature was proposed to be the cause of interference with the adherence of cell-surface and cell-cell [26,17,36]. The *B. licheniformis* compound reported here has also high content of phosphate groups and thus it can be proposed that the electronegative property of the compound might modulate the surface of the tested organism in such a way that there is a reduction or complete inhibition of the attachment of cell-surface or cell-cell.

It might be possible that the compound can modify the physicochemical characteristics and the architecture of the outermost surface of biofilm forming organisms which is the phenomenon observed for some antibiotics [37]. Reduction of cell surface hydrophobicity of *E. coli* PHL628 and *P. fluorescens* clearly indicates the modification of the cell surface, resulting in reduced colonization and thereby significant contribution to anti-biofilm effect. Almost similar results were obtained with coral-associated bacterial extracts for the anti-biofilm activity against *Streptococcus pyogenes* [14].

Anti-biofilm effects were reported to be accompanied in most cases by a loss of cell viability or the presence of quorum sensing analogues. Interestingly, the polysaccharide in the present study is devoid of antibacterial effect, which was demonstrated by the growth curve analysis and disc diffusion test with *E. coli* PHL628 and *P. fluorescens*. An almost similar observation has been reported with the exo-polysaccharide from the marine bacterium *Vibrio* sp. which displayed anti-biofilm nature without decreasing bacterial viability [17]. However, further experiments suggest that the present polysaccharide enhances the planktonic growth of *E. coli* PHL628 in the microtiter plate wells during biofilm production (data not shown). Another interesting phenomenon of the bioactive compound reported here is the absence of competition with the quorum sensing signals presumably present in supernatants of the target biofilm-forming bacteria used in this study. In addition, none of the previously reported quorum sensing competitors is structurally related to the polysaccharide reported here.

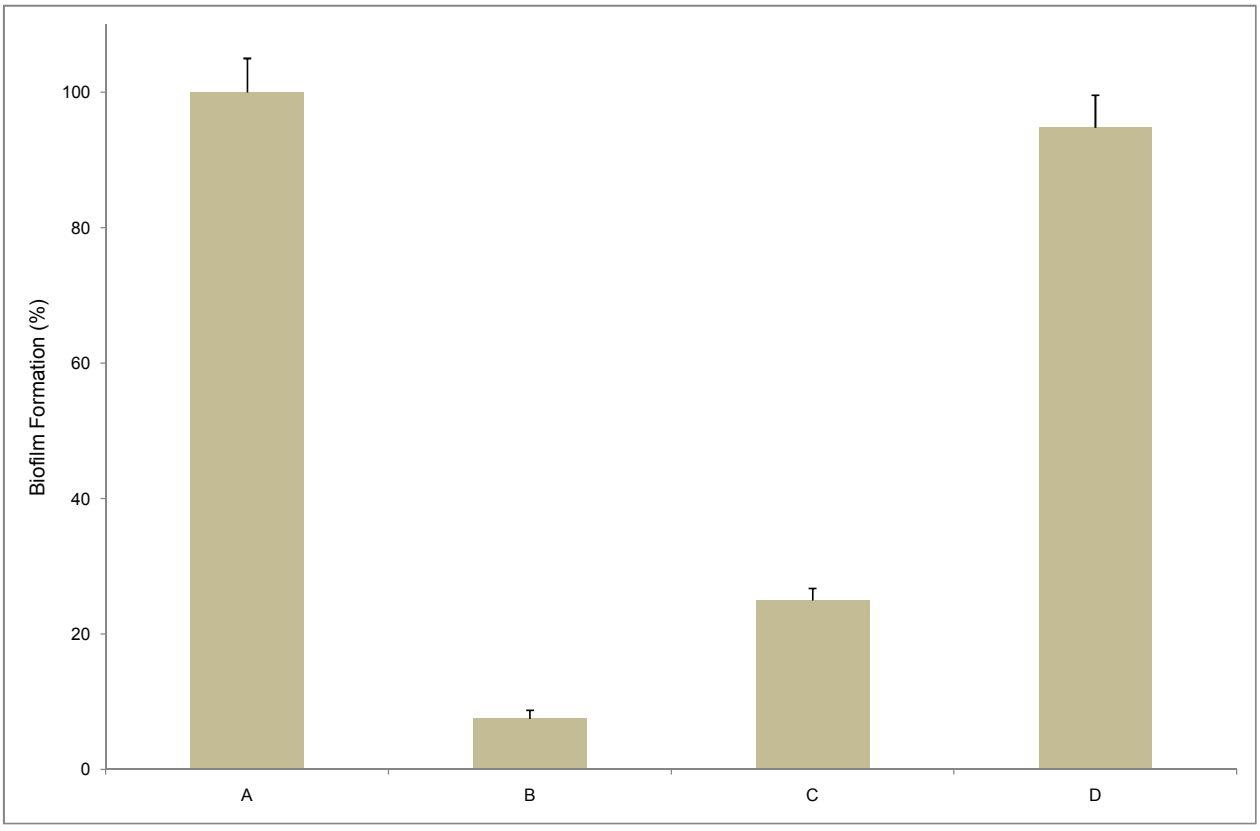

Figure 7 Pre-coating with the SP1 supernatant reduces attachment during biofilm formation. Biofilms of *E. coli* PHL628 were developed in 96-well microtiter plates in different conditions: no supernatant (A), wells pre-coated with supernatant (B), supernatant present (C), and supernatant added to pre-formed *E. coli* biofilm (D). The plate was incubated for 36 h, followed by crystal violet staining and spectrophotometric absorbance measurements (OD_{570}). The ratio of biofilm absorbance/planktonic absorbance was calculated, and this value is presented as the "biofilm formation" on the *y axis*. Bars represent means ± standard errors for six replicates.

In the cover slip experiment, biofilm inhibition was also evidenced and displayed a gradual decrease of biofilm development with the increase of the concentration of the polysaccharide in the culture of *E. coli PHL*628 and *P. fluorescens*. In addition, pre-coating the wells of the polystyrene microtiter plate with the compound also effectively inhibits biofilm formation. To our knowledge, coating with the polysaccharide from sponge-associated bacteria for inhibition of biofilm formation has been reported for the first time here, although there are some reports on the use of pre-coating surfaces with different surfactants and enzymes [38-41].

In conclusion, the polysaccharide isolated from sponge-associated *B. licheniformis* has several features that provide a tool for better exploration of novel anti-biofilm compounds. Inhibiting biofilm formation of a wide range of bacteria without affecting their growth represents a special feature of the polysaccharide described in this report. This characteristic has already been described for other polysaccharides in a few very recent articles [40-42]. Further research on such surface-active compounds might help developing new classes of anti-biofilm molecules with broad spectrum activity and more in general will allow to explore new functions of bacterial polysaccharides in the environment.

Methods

Isolation of bacterial strains

The bacterial strains used in this study were initially obtained from an orange-colored sponge, *Spongia officinalis*, collected from Mazara del Vallo (Sicilia, Italy), from a depth of 10 m. The sponge sample was transferred soon after collection to a sterile falcon tube and transported under frozen condition to the laboratory for the isolation of associated microbes. The sponge was then mixed with sterile saline water and vortexed. A small fraction of the liquid was serially diluted up to 10^{-3} dilutions and then spread on plates of Tryptone Yeast agar (TY). The plates were incubated at 37°C for 2 days till growth of colonies was observed. Single bacterial

colonies were isolated on the basis of distinct colony morphologies from the TY plates. Isolates were maintained on TY agar plates at 4°C until use.

Supernatant preparation

The isolated bacteria were sub-cultured on M63 (minimal medium) agar plates and incubated at 37°C for two days. A loopful of the bacterial culture from each plate was inoculated into M63 broth (in duplicate), incubated at 37°C for 24 h and then centrifuged at 7000× g for 20 minutes to separate the cell pellets from the fermentation medium. The supernatants were filtered through 0.2 μm-pore-size Minisart filters (Sartorius, Hannover, Germany). To ensure that no cells were present in the filtrates, 100 μl were spread onto TY agar plates, and 200 μl were inoculated in separate wells in the microtiter plate.

Screening for bioactive metabolites for biofilm inhibition

Filtered supernatants from the marine sponge-associated isolates were used to perform the assay for biofilm formation. The method used was a modified version of that described by Djordjevic *et al*. [43]. Overnight cultures of *E. coli* PHL628 strain grown at 37°C in $M63K_{10}$ broth (M63 broth with kanamycine, 10 μg ml^{-1}), were refreshed in $M63K_{10}$ broth and incubated again at 37°C for 5 to 6 h. 200 μl of inocula were introduced in the 96 well polystyrene microtiter plate with an initial turbidity at 600 nm of 0.05 in presence of the filtered supernatants from the different marine sponge associated isolates. The microtiter plate was then left at 30°C for 36 h in static condition.

To correlate biofilm formation with planktonic growth in each well, the planktonic cell fraction was transferred to a new microtiter plate and the OD_{570} was measured using a microtiter plate reader (*Multiscan Spectrum, Thermo Electron Corporation*). To assay the biofilm formation, the remaining medium in the incubated microtiter plate was removed and the wells were washed five times with sterile distilled water to remove loosely associated bacteria. Plates were air-dried for 45 min and each well was stained with 200 μl of 1% crystal violet solution for 45 min. After staining, plates were washed with sterile distilled water five times. The quantitative analysis of biofilm production was performed by adding 200 μl of ethanol-acetone solution (4:1) to de-stain the wells. The level (OD) of the crystal violet present in the de-staining solution was measured at 570 nm. Normalized biofilm was calculated by dividing the OD values of total biofilm by that of planktonic growth. Six replicate wells were made for each experimental parameter and each data point was averaged from these six.

Identification and purification of anti-biofilm compound

144 ml of cell free bacterial broth cultures were extensively dialyzed against water for two days, using a membrane tube of 12000-14000 cut-off; this procedure allowed us to remove the large amount of glycerol in the bacterial broth as confirmed by ^{1}H- ^{13}C-NMR experiments recorded on lyophilized broth before and after dialysis; the inner dialysate (25 mg) was fractionated by gel filtration on Sepharose CL6B, eluting with water. Column fractions were analyzed and pooled according to the presence of saccharidic compounds, proteins and nucleic acids. Fractions were tested for carbohydrate qualitatively by spot test on TLC sprayed with α -naphthol and quantitatively by the Dubois method [44]. Protein content was estimated grossly by spot test on TLC sprayed with ninhydrin and by reading the column fractions absorbance at 280 nm. The active fractions were tested by the Bio-Rad Protein System, with the bovine serum albumin as standard [45]. Finally, the presence of nucleic acids was checked by analysis of fractions absorbance at 260 nm. Furthermore, the grouped fractions were investigated by ^{1}H-NMR spectroscopy. ^{1}H and ^{13}C NMR spectra, were recorded at 600.13 MHz on a BrukerDRX-600 spectrometer, equipped with a TCI CryoProbeTM, fitted with a gradient along the Z-axis, whereas for ^{31}P-NMR spectra a Bruker DRX-400 spectrometer was used.

The gel filtration fractions were tested for anti-biofilm activity and the active fraction resulted positive to carbohydrate tests; this latter was a homogenous polysaccharide (6.6 mg) material. Preliminary spectroscopic investigations indicated the presence of a compound with a simple primary structure; the molecular mass of polysaccharidic molecule was estimated by gel filtration on a Sepharose CL6B which had previously been calibrated by dextrans (with a Mw from 10 to 2000 kDa). It's worthy to notice that some resonances in ^{13}C NMR spectrum (78.32, 70.76, 65.63, 67.15 ppm) were split; this suggested the presence of ^{31}P ($J_{C\text{-}P}$ from 4 to 9 Hz, see table 1) and its position into the polysaccharide repeating unit.

The phosphate substitution was confirmed by recording a ^{31}P-NMR spectrum; it showed a single resonance at 1.269 ppm [46].

The GC-MS analysis of the high-molecular-weight polymer was carried out on an ion-trap MS instrument in EI mode (70eV) (Thermo, Polaris Q) connected with a GC system (Thermo, GCQ) by a 5% diphenyl (30 m × 0.25 mm × 0.25 um) column using helium as gas carrier. Nuclear Overhauser enhancement spectroscopy experiments (NOESY) were acquired using a mixing time of 100 and 150 ms. Total correlation spectroscopy experiments (TOCSY) were performed with a spinlock time of 68 ms.

Heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) experiments were measured in the ^{1}H-detected mode via single quantum coherence with proton decoupling in the ^{13}C domain. Experiments were carried out in the phase-sensitive mode and 50 and 83 ms delays were used for the evolution of long-range connectivities in the HMBC experiment. The 2D ^{1}H-^{31}P HSQC experiment was recorded setting the coupling constants at 10 and 20 Hz.

Growth curve analysis

The effect of the bioactive compound on the planktonic culture was checked by growth curve analysis on both *E. coli* PHL628 and *Pseudomonas fluorescens*. The supernatant of the isolate was added to a conical flask containing 50 ml of M63 broth, to which a 1% inoculum from the overnight culture was added. The flask was incubated at 37°C. Growth medium with the addition of bacterial inoculum and without the addition of the supernatant was used as a control. OD values were recorded for up to 24 h at 1-h intervals.

Antibacterial activity by disk diffusion assay

Antimicrobial activity of the supernatant was assayed by the disc diffusion susceptibility test (Clinical and Laboratory Standards Institute, 2006). The disc diffusion test was performed in Muller-Hinton agar (MHA). Overnight cultures of *E. coli* PHL628 and *P. fluorescens* were subcultured in TY broth until a turbidity of 0.5 McFarland (1×10^{8} CFU ml^{-1}) was reached. Using a sterile cotton swab, the culture was uniformly spread over the surface of the agar plate. Absorption of excess moisture was allowed to occur for 10 minutes. Then sterile discs with a diameter of 10 mm were placed over the swabbed plates and 50 μl of the extracts were loaded on to the disc. MHA plates were then incubated at 37°C and the zone of inhibition was measured after 24 h.

Microscopic techniques

For visualization of the effect of the sponge-associated bacterial supernatant against the biofilm forming *E. coli* PHL628 and *Pseudomonas fluorescens*, the biofilms were allowed to grow on glass pieces (1 × 1 cm) placed in 6-well cell culture plate (*Greiner Bio-one, Frickenhausen, Germany*). The supernatant at concentrations ranging from 1 to 10 times was added in $M63K_{10}$ (for *E. coli* PHL628) and M63 broth (for *P. fluorescens*) containing the bacterial suspension of 0.05 O.D. at 600 nm. The wells without supernatant were used as control.

The plate was incubated for 36 h at 30°C in static condition. After incubation, each well was treated with 0.4% crystal violet for 45 minutes. Stained glass pieces were placed on slides with the bio-film pointing up and were inspected by light microscopy at magnifications of ×40. Visible bio-films were documented with an attached digital camera (*Nikon Eclipse Ti 100*).

Anti-biofilm effect on various strains and growth conditions

Some laboratory strains such as *Acinetobacter*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Salmonella typhimurium*, *Shigella sonneii*, *Listeria monocytogenes*, *Bacillus cereus*, *Bacillus amyloliquefaciens*, *Bacillus pumilus* and *Bacillus subtilis* were selected. All strains were grown in Tryptone Soya Broth (TSB) (Sigma) supplemented with 0.25% glucose and the same medium was used during the biofilm assay in the presence of SP1 supernatant.

Competitiveness between quorum sensing factors and bioactive compounds

For this experiment the *E. coli* PHL628 supernatant was prepared by using the same conditions as for that of the sponge-isolated strain. Equal volumes of the two supernatants were added either in combination or alone in the microtiter plate containing a culture of *E. coli* PHL628 at an initial turbidity of 0.05 at 600 nm and biofilm formation was measured as described above. Each result was an average of at least 6 replicate wells.

Pre-coating of microtiter plate

Wells were treated with 200 μl of the *B. licheniformis* supernatant for 24 h and then the un-adsorbed supernatant was withdrawn from the wells. Such pre-coated wells were inoculated with *E. coli* PHL628 cultures having an OD of 0.05 at 600 nm. In another set of wells that were not coated with the supernatant, the fresh culture of *E. coli PHL628* having the same density mentioned above were added together with the supernatant (5% v/v). The microtiter plate was then incubated for 36 h in static conditions and biofilm formation was estimated. The control experiments were carried out in wells that were not pre-coated or initially added with the supernatant. Each result was an average of at least 6 replicate wells and three independent experiments.

In a parallel microtiter plate, the supernatant was added to the 36-h biofilm culture in the microtiter plate and was then left at 30°C in static conditions for another 24 h. The experiment was repeated six times to validate the results statistically.

Microbial cell surface hydrophobicity (CSH) assay

Hydrophobicity of the culture of *E. coli* PHL628 and *P. fluorescens* were determined by using MATH (microbial adhesion to hydrocarbons) assay as a measure of their adherence to the hydrophobic hydrocarbon (toluene) following the procedure described by Courtney *et al*.

2009 [47]. Briefly, 1 ml of bacterial culture (OD530 nm = 1.0) was placed into glass tubes and 100 μl of toluene along with the supernatant (5% v/v) was added. The mixtures were vigorously vortexed for 2 min, incubated 10-min at room temperature to allow phase separation, then the OD530 nm of the lower, aqueous phase was recorded. Controls consisted of cells alone incubated with toluene. The percentage of hydrophobicity was calculated according to the formula: % hydrophobicity = [1-(OD530 nm after vortexing/OD530 nm before vortexing)]×100.

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Authors' contributions
MV planned the work that led to the manuscript; SMAS produced and analyzed the experimental data; AZ, AC and MDF participated in the interpretation of the results; MV, SMAS and MDF wrote the paper; EM, LC and AT performed the chemical characterization of the bioactive compound. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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